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Direktor: Prof. Dr. Kurt Bürki

Arbeit unter Leitung von Dr. Paolo Cinelli

Reprogramming of Rat Embryonic Fibroblasts Derived From Different Rat Strains and Function of Pramel7 in Reprogramming

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Jon Paulin Zumthor

Tierarzt
von Haldenstein, Schweiz

genehmigt auf Antrag von

Prof. Dr. Kurt Bürki, Referent

Prof. Dr. Max Gassmann, Korreferent

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Summary

In 2006 Yamanaka and colleagues succeeded to convert somatic cells into induced pluripotent stem cells. To do so, they introduced four defined transcription factors, Oct-3/4, Sox2, Klf4 and c-myc, by retroviral infection.
This methodology opens a new field in stem cell research, not only in terms of future medical applications but also for new approaches in gene targeting in animals where no stable ES cells are yet available like the rat.

The three goals of this work were: Firstly, the determination of differences in reprogramming efficiency in dependence of the genetic background of the rat cells used. Secondly, the generation efficiency of rat iPS (riPS) cells from wild type rat embryonic fibroblasts (REFs) before and after gene targeting. The third goal was to clarify the role of Pramel7, a protein that was recently shown to stabilize the pluripotent state of embryonic stem cells, in terms of reprogramming efficiency.
For all reprogramming rounds 3 factors (Oct-3/4, Klf4, SOX2) were used.

By reprogramming REFs of the different genetic backgrounds no difference in the reprogramming efficiency could be obtained.
The generation efficiency of riPS before and after gene targeting was similar, indicating that gene targeting does not affect the reprogramming potential.
The results obtained in this work regarding the role of Pramel7 in
reprogramming, showed that Pramel7 overexpression is sufficient to drive reprogramming in a LIF independent manner.

Zusammenfassung

2006 wurde eine neue Technologie vorgestellt, entwickelt von Yamanaka und Mitarbeitern, welche es erlaubt, somatische Zellen zu stammzellartigen Zellen zu reprogrammieren. Um dies zu erreichen, wurden bestimmte Transkriptionsfaktoren (Oct-3/4, Sox2, Klf4, c-myc), unter Verwendung von Retroviren, in die Zielzellen eingeführt. Diese Methode eröffnet neue Möglichkeiten für die Stammzellforschung, nicht nur bezüglich möglichen medizinischen Anwendungen, sondern auch für neue Ansätze zur Erzeugung genetisch modifizierter Tiere, für die keine stabilen ES Zellen verfügbar sind, wie das für die Ratte der Fall ist.
Zwei Ziele dieser Arbeit waren, zu ermitteln, ob der genetische Hintergrund der zu reprogrammierenden Rattenzellen, oder eine vorangegangene genetische Manipulation derselben, einen Einfluss auf die Effizienz des Reprogammierungprozesses hat. Ein drittes Ziel war es, die Rolle von Pramel7, einem Protein mit nachgewiesener pluripotenzstabilisierender Wirkung, in Bezug auf die Reprogrammierungseffizienz zu analysieren.

Bei der Reprogrammierung von Rattenzellen unterschiedlicher genetischer Herkunft konnten keine Unterschiede bezüglich Reprogrammierungseffizienz beobachtet werden.
Genetisch manipulierte und WT Rattenzellen zeigten keinen Unterschied im Reprogrammierungsprozess.
Die Resultate bezüglich der Rolle von Pramel7 im Reprogrammierungsprozess konnten zeigen, dass eine Überexpression von Pramel7 eine von LIF unabhängige Reprogrammierung erlaubt.

Abbreviations

2i	Dual inhibitors
AP	Alkaline Phosphatase
APC	Adenomatous polyposis coli
BIO	6-bromindirubin-3'-oxime
BMP	Bone morphogenetic protein
BN	Brown Norway
c-myc	Myelocytomatosis oncogene
DNA	Deoxyribonucleic acid
E13.5	Embryonic day 13.5
ERK	Extracellular signal-regulated protein kinase
ES cells	Embryonic stem cells
G418	Geneticin
GSK3	Glycogen-synthase kinase-3
hES cells	Human embryonic stem cells
HMG	High-mobility group
hsp90	Heat-shock-protein 90
ICM	Inner cell mass
id	Infection day
IHC	Immunohistochemistry
IL	Interleukin
iPS cells	Induces pluripotent stem cells
IRES	Internal ribosomal entry site
JAK	Non-receptor tyrosine kinase Janus
Klf4	Kruppel-like factor 4
KO	Knock-out
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
Mek	Mitogen activated protein kinase
MER	Modified-oestrogen-receptor
mES cells	Mouse embryonic stem cells
min.	Minutes
mRNA	Messenger ribonucleic acid
NP cells	Neural progenitor cells
NS cells	Neural stem cells
o.n	Over night
Oct-3/4	Octamer-binding transcription factor –3/4
OHT	4-Hydroxytamoxifen
PB	PiggyBac
PBS	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
PRAME	Preferentially expressed antigen in melanoma

rFb-medium	Rat Fibroblast medium
riPS cells	Rat induced pluripotent stem cells
RNA	Ribonucleic acid
SD	Sprague Dawley
SDxBN	Sprague Dawley x Brown Norway crossing
Sox2	SRY-related HMG-box gene 2
SSEA-1	Stage-specific embryonic antigen-1
STAT3	Signal transducer and activator of transcription-
TIK	Anti- phosphotyrosine immunoreactive kinase
VPA	Valporic acid
WT	Wildtype

A. Introduction

1. Characterization of pluripotent stem cells

The ability to differentiate into cells of all three germ layers and the capacity of maintaining proliferative state for an indefinite time period, are two main characteristics of pluripotent stem cells, and are of great interest in regenerative medicine, drug development and developmental biology research.

More precisely, pluripotent stem cells should match the following criteria. First, they should possess the ability to proliferate indefinitely in vitro and thereby maintain an intact diploid karyotype. Second, they need to be capable of in vitro differentiation into cells of all three germ layers (ectoderm, mesoderm, endoderm). Third, pluripotent stem cells should express pluripotency markers, such as, stage-specific embryonic antigen-1 (SSEA-1), octamer-binding transcription factor –3/4 (Oct-3/4) or Nanog. Fourth, they should form teratoma, containing cells of all three germ layers, when injected subcutaneously into immuno-incompetent mice. Last but not least the true "gold standard" test for pluripotency is to introduce stem cells into a blastocyst, in order to demonstrate their capacity to contribute to the formation of chimera and germ cells.

Pluripotent stem cells can be obtained from different sources:

Embryonic stem (ES) cells, obtained from the inner cell mass (ICM) of the blastocyst, are the best-known pluripotent stem cells (Evans and Kaufman, 1981; Martin, 1981). In 1968 Gardner et al. demonstrated the pluripotent character of the ICM. By injecting cells derived from the ICM of a blastocyst into another and then transferring the blastocyst into a foster mother, they were able to generate chimeric animals (Gardner and Edwards, 1968).

Mouse spermatogonial stem cells were shown to be induced into ES-like pluripotent cells under certain in vitro culture conditions (Guan et al., 2006; Kanatsu-Shinohara et al., 2004). However, this method is only exercisable in male and has shown to cause tumorigenesis (Hochedlinger and Jaenisch, 2006).

Alternative sources for pluripotent stem cell were revealed by the development of different techniques to reprogram somatic cells into an embryonic state either by cell fusion or by nuclear transfer.

To reprogram a somatic cell by nuclear transfer, a somatic nucleus is injected into an enucleated oocyte (Figure 1). The first cloned animal with this technique was in 1997 the sheep Dolly (Wilmut et al., 1997). Other laboratories successfully cloned mice from lymphoid cells (Hochedlinger and Jaenisch, 2002; Inoue et al., 2005) and from postmitotic neurons (Eggan et al., 2004; Li et al., 2004). These results indicate, that the potential of the nucleus is not restricted by terminal differentiation (Jaenisch and Young, 2008). However, this method is inefficient due to faulty reprogramming and most of the clones die soon after implantation or are born with abnormalities (Jaenisch and Young, 2008).

The fusion of somatic cell with an ES cell leads to epigenetic reprogramming of the somatic nucleus to undifferentiated state (Fig. 1). Several groups could prove this method by the production of murine hybrids, by fusion somatic cells and embryonic stem cells. The cell fusion assay was successfully per-

formed with embryonic germ cells, ES cells (Zwaka and Thomson, 2005) and with embryonic carcinoma cells (Solter, 2006). The resulting hybrids of these studies share many characteristics with parental ES cells, which demonstrate that the pluripotent phenotype is dominant in such cell fusion products (Jaenisch and Young, 2008). However, the cell fusion technique has to deal with low efficiency and the tetraploid character of the reprogrammed cell, in turn preventing the usage of these cells for customized therapy.

The most auspicious method to reprogram somatic cells to pluripotent a state was developed by Yamanaka and collaborators. Due to the fact that transferring a somatic nucleus into an enucleated oocyte as well as the fusion of a somatic cell with ES cells can lead to the reprogramming of a somatic cell, they presumed that ES cells and unfertilized eggs contain factors sufficient to reprogram somatic cells. In 2006 Yamanaka and Takahashi could successfully reprogram mouse embryonic fibroblasts (MEFs) to pluripotent ES-like cells, termed "induced pluripotent stem cell" (iPS), by using only four transcription factors (Fig. 1), the so called Yamanaka factors.

The development of iPS cells is based on the research on ES cells; therefore, the next part of this introduction will present the establishment of ES cells and the pathways and genes involved in the maintenance of their pluripotency. In the last part, the identification of the Yamanaka factors and alternative methods for the generation of iPS cells, are described.

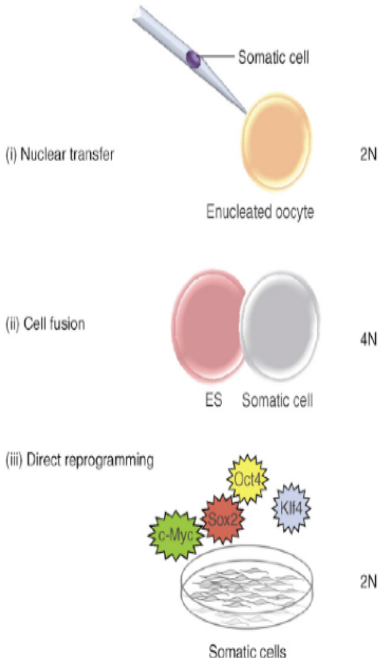


Fig. 1: Methods to induce nuclear reprogramming. **(i)** Through the injection into an enucleated oocyte, somatic cells can be reprogrammed (nuclear transfer). **(ii)** The fusion of a somatic cell with a pluripotent cell, leads to a tetraploid pluripotent cell (cell fusion). **(iii)** The ectopically expression of the four transcription factors, mediated by viral integration, is sufficient to bring somatic cells to a pluripotent state (direct reprogramming) (from Amabile, 2009).

1.1. Mouse embryonic stem cells

Derived from the ICM of a preimplatation blastocyst, mouse embryonic stem (mES) cells are pluripotent. Therefore, they possess the ability to differentiate into all tissues of the developing embryo, including the germ cells (Martin, 1981) (Evans and Kaufman, 1981). When the morula stage in murine embryo development is reached, the cells of the morula start to differentiate into two different cell lineages. One lineage is competent to form all extraembryonic tissues, including the placenta. The other lineage is responsible to form the ICM, which will form the whole embryo. At embryonic day 4.5 the ICM differentiates into primitive ectoderm, giving rise to all somatic tissues and germ cells, and into primitive endoderm, which contributes, to the yolk sac (Manipulating the mouse embryo, Nagy et al., 2003).

In 1981, two research groups described the successful cultivation of pluripotent cell lines from mouse blastocyst. Martin et al. could cultivate these cells by using ES cell conditioned medium containing a "growth factor" avoiding differentiation, while Evans and Kaufmann made first a feeder layer with MEFs and then seeded the ES cells on this layer (Evans and Kaufman, 1981; Martin, 1981). The cells discovered by these two groups could be kept in culture without losing any differentiation potential. To demonstrate the pluripotency of these cells, further experiments were done. When injected into a blastocyst, live mouse chimeras were born and ES cells showed their competence to contribute to all cell lineages including germ cells (Bradley et al., 1984).

Also in vitro differentiation of ES cells into cells of all three germ layers was successfully performed (Fig. 2) (Doetschman et al., 1985; Evans and Kaufman, 1981; Wobus et al., 1984)

Nevertheless, the molecular mechanism linked to stabilisation and differentiation of ES cells from different mouse strains are little known to date. Among mouse strains, genetic background strongly affects the efficiency of ES cells isolation and almost all ES line in use are derived from the strain 129. ES cells of proven ability to colonize the germ line have been obtained at very low frequency in only a few mouse strains other than 129 (Simpson et al., 1997). Only after adjusting the culture conditions, germ line competent inbred ES cells could be established, e.g. from C57BL/6 (Ledermann and Burki, 1991), DBA/1lacJ (Roach et al., 1995), and BALB/c mice (Noben-Trauth et al., 1996).

Because of the finding of Evans and Kaufmann, that the generation of ES cells can only be established, when cultivated on a feeder layer, it was assumed that fibroblast secrete some critical factors. It was suggested that these factors are responsible to promote self-renewal or to suppress differentiation. Two groups then identified independently leukaemia inhibitory factor (LIF) in 1988 to be the factor responsible for maintaining pluripotency or to suppress differentiation (Smith et al., 1988; Williams et al., 1988). LIF is a glycoprotein and a member of the interleukin (IL)-6 family of cytokines. Through signal transduction and activation of transcription (STAT) signalling, LIF regulates many cell functions via membrane-bound gp130 signalling complex (Burdon et al., 1999). Since its discovery, LIF is directly added to the culture medium. LIF alone is not sufficient to keep ES cells pluripotent *in vitro*. The cells also require the presence of fetal bovine serum in the culture medium, FBS contains several

growth factors necessary for the ES cells. In serum-free ES cell culture conditions, LIF is only able to inhibit differentiation and maintain pluripotency, when bone morphogenetic protein (BMP) is added to the medium. In combination with BMP, LIF perpetuates self-renewal, chimera contribution and germline transmission properties (Wobus and Boheler, 2005).

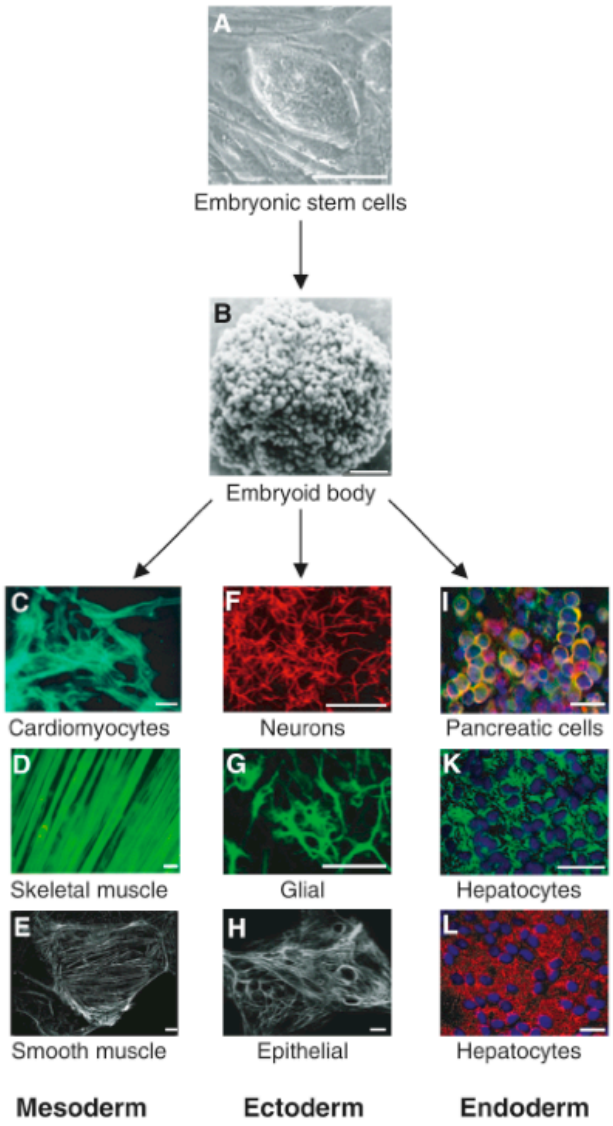


Fig. 2: Murine ES cell lines were shown to have the in vitro-potential to generate many cell types of all three germ layers. Undifferentiated ES cell clonoies **(A)** are aggregated to three dimensional cell clumps (embryoid bodies) **(B)** that further develop into different cell types **(C-L)** (from Wobus and Boehler, 2005).

Round shape and shiny borders characterize the morphology of ES cells in culture (Fig. 3). To demonstrate the differentiation potential of ES cells it is possible to inject them subcutaneously into immune-deficient mice and produce teratoma-containing tissues from all three germ layers. *In vitro*, it is possi-

ble to generate aggregates of ES cells, so called embryoid bodies, which have the ability to differentiate into different cell types upon stimulation with specific growth factors.

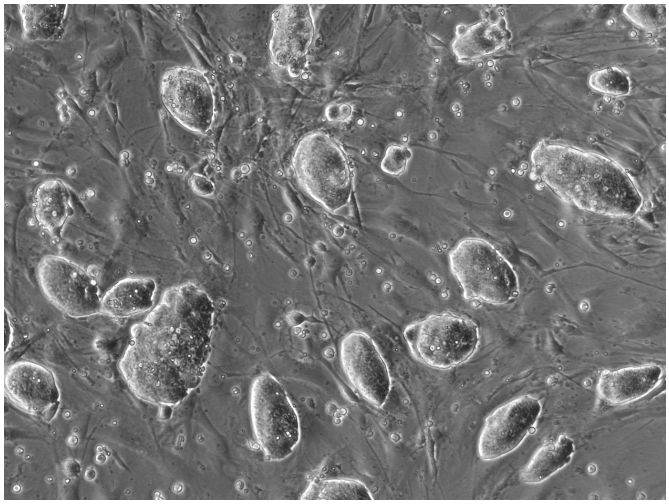


Fig. 3: ES cells in culture are forming bright round colonies with shiny borders (from Casanova, 2008).

2. Signalling pathways involved in maintenance of pluripotency

The development of a whole mammalian organism requires the specification of over 200 cell types from a single totipotent cell. It is fundamental to understand the regulatory networks that are responsible for the pluripotency of ES cells. Different pathways are known to modulate stem-cell pluripotency:

2.1. The LIF signalling cascade

The fact that LIF sustains ES cells only when serum is added, reveals that additional factors are required. LIF as an exogenous factor binds to its cell-surface receptor, the LIF receptor (LIFR)-gp130 heterodimeric receptor. Once bound at the receptor, it activates the signal transducer and activator of transcription-3 (STAT3), which undergoes phosphorylation and dimerization (Fig. 4)(Niwa et al., 1998). After dimerization, the STAT3 dimers translocate to the nucleus where they act as transcription factors.

To maintain pluripotency without LIF, one can use a conditionally active form of STAT3. The STAT3-modified-oestrogen-receptor (MER) fusion protein can be induced by addition of hydroxytamoxifen to the medium. In the absence of hydroxytamoxifen, the STAT3-MER fusion protein is constantly translated, but its activity is repressed by binding to heat-shock protein 90 (hsp90). Addition of hydroxytamoxifen causes dissociation of hsp90 from STAT3-MER, resulting in STAT3 activation.

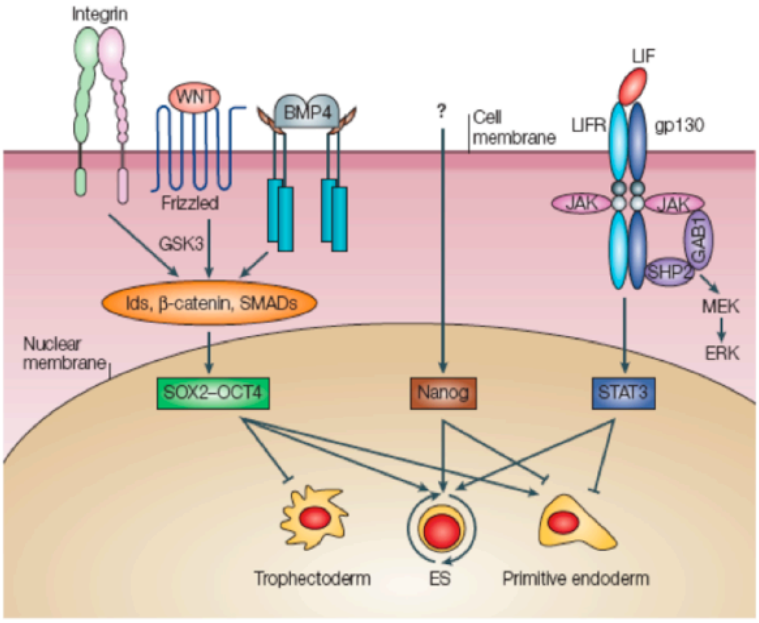


Fig. 4: Binding of extracellular ligands such as BMP4 or LIF induces intracellular signalling cascades resulting in recruitment of pluripotency controlling transcription factors as Stat3, Nanog or Oct-3/4. Activation of Oct-3/4 via BMP4 and Wnt-signalling and Stat3 via LIF and Jak/Stat-pathway are well known, whereas Nanog recruitment mechanisms are still poorly understood (from Boiani, 2005

Nevertheless, when STAT3-MER is expressed at a low level, it is not able to maintain the fully pluripotent state. These findings lead to the presumption, that a minimal level of STAT3 is required for the propagation and pluripotency of ES cells (Matsuda et al., 1999).

Besides the STAT3-pathway, LIF can also activate other pathways by recruiting the non-receptor tyrosine kinase Janus (JAK) and the anti-phosphotyrosine immunoreactive kinase (TIK). Another feature of LIF is to induce the phosphorylation of extracellular signal-regulated protein kinases, ERK1 and ERK and thus increase mitogen-activated protein kinase (MAPK) activity, which was shown to impair self-renewal (Boeuf et al., 1997; Burdon et al., 1999). This is mediated through activation of the widely expressed tyrosine Src-homolgy-2 (SH2)-Domain-containing protein tyrosine phosphatase-2 (SHP-2)(Auernhammer et al., 2000).

The LIF/STAT3 pathway is not universal because of its different effects in human and mouse ES cells, and might not be crucial for self-renewal in human embryonic stem (hES) cells, as Oct-3/4 is down regulated in hES cells, even when human derived LIF is used (Sato et al., 2004).

2.2. The signal protein bone morphogenetic protein 4

The exact role of BMP4, related to the molecular scheme of pluripotency, is not fully understood yet. It is thought to be a key anti-neurogenesis factor in the embryo. *In vitro*, ES cells lose pluripotency and differentiate into neurons when BMP4 is not present. In ES cell culture, BMP4 supports self-renewal and the maintenance of pluripotency, when LIF is present. It activates the gene encoding the transcription factor SMAD4 (similar to mothers against decapentaplegic homologue-4). SMAD4 then activates genes from the inhibitor of differentiation gene family. Finally the inhibitor of differentiation genes recruit the Oct-3/4, which acts in connection with SRY-related high-mobility (HMG)-box protein-2 (Sox2) and other cofactors, activating or repressing expression of pluripotency-related genes in the nucleus (Fig. 4) (Boiani and Scholer, 2005). If BMP4 is used to cultivate ES-cell in absence of LIF, it activates other SMAD transcription factors, which inhibit the Id-genes and therefore counteracts the activity of the LIF cascade. Taken together, BMP4 is most likely the factor delivered by the serum in ES-cell cultures containing serum and LIF (Ying et al., 2003). In conclusion, the balance between LIF and BMP4 and their interaction with the cell surface, seem to be responsible for maintaining the undifferentiated state of mES cells.

2.3. Wnt proteins

Wnt proteins are secreted glycoproteins that have various roles in tissue differentiation and organogenesis (Cadigan and Nusse, 1997). On one hand, the Wnt signalling pathway has been implicated in maintenance of pluripotency of stem cells, and on the other hand it plays a role in the induction of specific tissues and development of organs and it is often ectopically activated in tumours. 19 different Wnt genes encoding for secreted proteins, which signal by binding to their receptors are known. The best known Wnt pathway is the canonical pathway. It is activated upon binding of the Wnt proteins to the Frizzled receptor on the cell membrane (Fig. 5).

After a series of phosphorylation steps, the pathway leads to inhibition of glycogen-synthase kinase-3 (GSK3), and subsequent stabilisation of the nuclear import of β-Catenin and the expression of target genes e.g. proto-oncogene myc and other growth factors. The possible involvement of the Wnt pathway in the short-term maintenance of hES cells and mES cells has been reported. Two groups could show that β-Catenin is localised in the nuclei of the ICM cells but not in the trophoblast cells in the blastocyst (Sato et al., 2004; Wang et al., 2004). These findings lead to the assumption, that Wnts may be involved in cell determination in the preimplantation embryo. To demonstrate that the activation of the canonical pathway has the competence to maintain the undifferentiated state in hES cells and mES cells and to perpetuate the expression of Oct-3/4, REX1 (also known as zinc-finger protein-42; ZFP42) and Nanog, even after removal of LIF, Sato et al. used a specific reversible inhibitor of GSK3, 6-bromindirubin-3'-oxime (BIO). The inhibition of GSK3 with BIO leads to permanent activation of the Wnt pathway and the maintenance of the undifferentiated state in hES cells and mES cells as well as to the retention of the expression of Oct-3/4, Nanog and REX1. The removal of the inhibitor leads

to normal differentiation in hES cells and mES cells (Sato et al., 2004). Gene manipulation of Wnt signalling in ES cells has been performed. Haegele et al. performed two experiments with the same result. Inactivating of the adenomatous polyposis coli (APC) complex or the introduction of a dominant-active form of β-Catenin, both manipulations lead to inhibition of neural differentiation *in vitro* (Haegele et al., 2003).

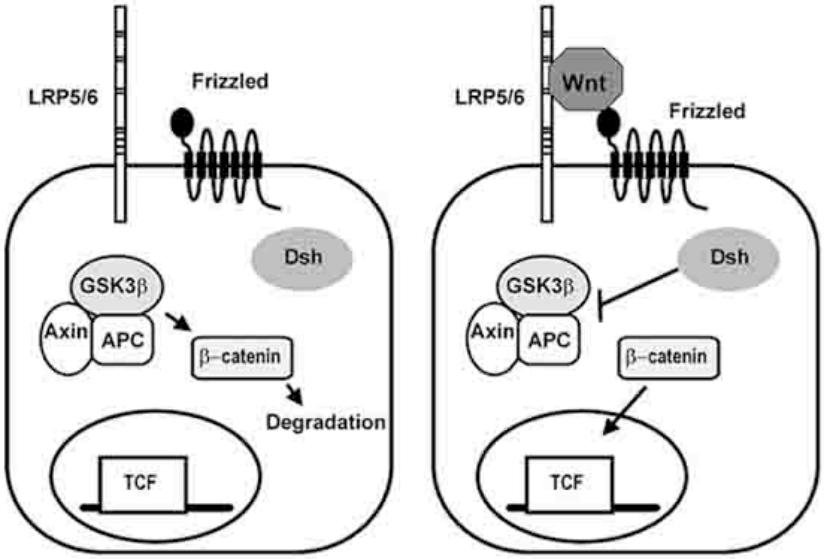


Fig 5: The canonical Wnt signal pathway. Left, in absence of Wnt ligands, little β-Catenin is present in the cytoplasm because it is degraded as a result of phosphorylation by Axin/APC/GSK3beta complex; Right, Interaction of Wnt with Frizzeld and LRP5/6 receptors activates Dishevelled (Dsh) wich in turn inactivates the

Even though there is evidence of the involvement of Wnts in pluripotency and self-renewal, their exact role is still unclear and further research has to be done.
Besides other pluripotency related genes (e.g. Oct-3/4 or Stat3), there are other two genes whose role in maintaining pluripotency is not well understood yet:
The pluripotency related genes Nanog and Pramel7.

3. Nanog

In 2003 Chambers et al. and Mitsui and colleagues described Nanog as a novel divergent homeobox-containing transcription factor which functions as an intrinsic determinant for self-renewal and prevention of differentiation in ES cell independently of the LIF/Stat3 pathway and called it Nanog, after the mythological Celtic land of the ever young “Tir nan Og”(Chambers et al., 2003; Mitsui et al., 2003).
Nanog seems to cooperate together with Oct-3/4 and Sox2 for the establishment of the identity of ES cells. The Nanog protein is absent during early cleavage and is first apparent in the interior of the compact morula and later on in the ICM of the blastocyst. Nanog is thought to regulate the maintenance of pluripotency, prevent differentiation and the cell fate of the epiblast, since the embryo fails to develop in Nanog deleted mice (Chambers et al., 2003; Loh et al., 2006; Mitsui et al., 2003).

Although previous analysis suggested that a transient downregulation of Nanog would cause differentiation (Mitsui et al., 2003), newer findings describe that Nanog is dispensable for expression of somatic pluripotency (Chambers et al., 2007). In the permanent absence of Nanog, ES cells appear to predispose toward differentiation but still can self-renew indefinitely. Therefore, they are not making any commitment in direction of a cell lineage.
These findings show Nanog as the responsible gene for germ cell formation, assembling of ICM and assumably for stabilization of ES cells rather than being directly involved in the housekeeping machinery of pluripotency (Chambers et al., 2007).

4. Pramel7

Analysis of Oct-3/4 related genes, revealed Pramel7 as pluripotency related gene showing no expression in somatic tissues of the mouse but in pluripotent tissue from cleavage stage embryos to the epiblast of late blastocyst and embryonic germ cells (Bortvin et al., 2003).
The fact, that Pramel7 is only transcribed in pluripotent tissues suggests that it may play a role in early embryo development.
The pattern of its expression is similar to the one from Nanog (Chambers et al., 2003). In 2008 Cinelli et al. showed that when Pramel7 is overexpressed in ES cells, it is capable to keep embryonic stem cells in a pluripotent state in a LIF independent manner, as the overexpression of STAT3 or Nanog does. Pramel7 is up regulated in STAT3 overexpressing cells (Cinelli et al., 2008). PRAME (preferentially expressed antigen in melanoma) antigen and Pramel7, they encode for similar proteins. Therefore the name PRAME like 7. PRAME is known to inhibit retinoic acid-induced differentiation (Bortvin et al., 2003; Epping et al., 2005). The role of Pramel7 in embryo development and its regulation are unknown. Nevertheless, its expression pattern and its ability to maintain pluripotency in absence of LIF when overexpressed, indicates an important role of Pramel7 in maintaining pluripotent ES cells.

5. Induced pluripotent stem cells

Due to ethical issues concerning the use of embryos for derivation of ES cells, the development of iPS cells from somatic cells in 2006 by the Yamanaka group, was an important step in terms of clinical applications.
iPS cells are derived from somatic cells by ectopic expression of few transcription factors. iPS cells share many features with ES cells derived from the ICM of a blastocyst. Like ES cells, iPS cells are able to self renew indefinitely and differentiate into all types of cells in the body.
The reprogramming technique developed by the Yamanaka group also offers new possibilities to establish gene targeting in the rat, as it is used in the mouse over years. Compared to the mouse, rat as a model of human diseases offers many advantages. Since it was once the most widely used organism in medical research, a lot of data has already been generated, in addition, the physiology of the rat is eas-

ier to monitor and is more corresponding to the human organism. Because of its bigger size, the performance of surgical techniques is enhanced and its organs are easier to examine.
The next chapter talks about the history of iPS cells and shortcomings in generation of iPS cells.

5.1. Identification of the Yamanaka-factors

24 genes, which play an important role in the induction and maintenance of pluripotency, were selected. To elucidate the potential capacity of each single gene in reprogramming somatic cells, the authors developed an assay system to detect the pluripotent state of a reprogrammed cell (Fig. 6).

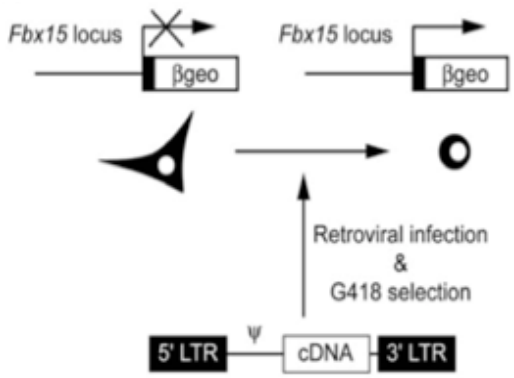


Fig.6: The Yamanaka/Takahashi-assay: Candidate gene cDNAs were introduced into the Fbx15$^{\beta geo/\beta geo}$-locus. Selection of pluripotent colonies was mediated by resistance to G418 (from Takahashi, 2006)

They inserted a βgeo cassette (containing β-galactosidase and neomycin resistance genes) into the mouse Fbx15 gene by homologous recombination. Based on the fact, that the mouse Fbx gene is expressed in ES cells and early embryos, and that it is not necessary for the maintenance of pluripotency nor embryo development (Tokuzawa et al., 2003), the pluripotent state of a cell could be detected as resistance to G418 (Geneticin).
They then introduced each of the 24 selected candidate genes into MEFs, homozygous for the βgeo knock-in construct (Fbx 15$^{\beta geo/\beta geo}$), by retroviral transduction. By culturing those transduced cells in ES cell medium containing G418, they did not observe drug resistant colonies. With the introduction of all 24 candidate genes together, drug resistance colonies could be generated. To identify which of the 24 candidate genes were critical, they sequentially repeated the transduction of the retroviruses containing the candidate genes by omitting individual factors from the pool of the candidate genes, and analyzed the effect on the formation of G418-resistant colonies. With this test arrangement, 10 factors were identified, whose removal resulted in no colony formation after 10 days. In a next step, the effect of withdrawal of individual factors from those 10 factors, relating to colony formation was analyzed. The removal either of Oct-3/4 or kruppel-like factor 4 (Klf4) resulted in no colony formation. When Sox2 was withdrawn, only a few drug-resistant colonies grew. Without myelocytomatosis oncogene (c-myc) only flatter colonies with non-ES-cell-like morphology appeared. The withdrawal of the remaining 6 factors had no influence on colony formation.
These results demonstrated the important role of Oct-3/4, Klf4, Sox2 and c-myc for the reprogramming process of MEFs (Takahashi and Yamanaka, 2006).
The generated iPS cells with 4 factors were positive for the majority of the marker genes for pluripotency. Besides the positive staining of these colonies for alkaline phosphatase (AP) and SSEA-1, they also showed high telomerase activity. When injected into immuno-incompetent mice, the iPS cells

gave rise to teratomas that after histological examination were shown to contain cells from all three germ layers.

Takahashi and Yamanaka also succeeded to generate iPS cells from adult fibroblasts derived from tail-tips of Fbx 15$^{\beta geo/\beta geo}$ mice. As the iPS from MEFs, the iPS generated from adult fibroblasts, were positive for most of the ES-cell marker genes, e.g. Sox2, Oct-3/4 and Nanog. They had the ability to form teratomas containing tissue from all three germ layers and were positive for AP. When injected into a blastocyst, iPS cells contributed to mouse embryonic development.

The finding of Yamanaka and Takahashi is an important step in understanding the processes controlling pluripotency. However, the exact mechanisms of how the four factors are mediating reprogramming are not well understood yet, but the role of the single factors was already extensively analyzed previously:

5.1.1. Oct-3/4

Oct-3/4 is a transcription factor containing the POU-domain. It was first discovered as a protein expressed in ES cells, unfertilized oocytes and primordial germ cells and is used as a pluripotency marker (Scholer et al., 1989).

Nichols et al. (1998) showed that the expression of Oct-3/4 is necessary for the development of the inner cell mass in vivo, thus for the derivation of ES cells as well as for maintaining pluripotency. They also showed, that Oct-3/4 deficient mouse embryos only develop to a blastocyst like stage, but instead of ICM cells, only cells showing trophoectodermal characteristics can be observed (Nichols et al., 1998).

The expression level of Oct-3/4 in ES cells is responsible for stipulating three different cell destinies. ES cells retain their undifferentiated and pluripotent state, when the expression level is within a narrow window. Less than a twofold increased expression level of Oct-3/4 makes the cells differentiate into primitive endoderm and mesoderm. In contrast, down regulation causes loss of pluripotency and differentiation into trophoectoderm (Niwa et al., 2000). Therefore a precise expression level of Oct-3/4 is needed to keep ES-cell in a pluripotent state. It is thought to prevent cells from the ICM to differentiate into throphoectoderm and perhaps also into somatic cell lineages.

5.1.2. Sox2

SRY (sex determining region Y)-box 2, known as Sox2 is a member of the HMG-domain DNA-binding-protein family. Sox2 acts as a transcription factor and is involved in the regulation of the chromatin structure (Pevny and Lovell-Badge, 1997). Sox2 heterodimerizes with Oct-3/4 and plays a pivotal role in maintaining pluripotency in ES cells. It is not only expressed in ES cells, but also in the extra-embryonic ectoderm, in trophoblast stem cells and in neural stem (NS) cells (Avilion et al., 2003; Masui et al., 2007). When Sox2 is inactivated by gene targeting, developing embryos show a defective primitive ectoderm. However these Sox2$^{-/-}$ blastocysts can be rescued by the injection of wild-type ES cells (Avilion et al., 2003). Boyer et al. found that several binding sites from Sox2 and

Oct-3/4 are overlapping in human ES cells and that 87% of those sites overlaps with Nanog targets (Boyer et al., 2005). Summarizing these data give rise to the hypothesis, that the combined expression of Oct-3/4, Sox2 and Nanog specifies the first three cell lineages that arise soon after implantation.

5.1.3. Klf4

The transcription factor Klf4 is expressed in many different tissues, e.g. skin, kidney, the epithelium of the intestine and murine ES cells (Segre et al., 1999; Yamanaka, 2008). Klf4 is able to either activate or repress transcription depending on the target gene and it's interaction partners (Rowland and Peeper, 2006). Another attribute of Klf4 is to act both as tumor suppressor and as oncogene (Evans and Liu, 2008; Zhao et al., 2004). If Klf4 is constitutively expressed, it suppresses cell proliferation by blocking G_1-S progression of the cell cycle. But in human colorectal carcinoma cells a down regulation of Klf4 was described (Zhao et al., 2004). Li et al. demonstrated in 2005, that when Klf4 is overexpressed in ES cells, it avoids differentiation in erythroid progenitors, suggesting a role for this factor in ES cell function (Li et al., 2005). However, it is not fully understood what the exact role of Klf4 in the reprogramming process is and if it is exchangeable with other Klf family members, such as Klf2 and Klf5, or the unrelated factors Nanog and Lin28 (Nakagawa et al., 2008; Yu et al., 2007).

5.1.4. c-myc

c-myc acts as a pleiotropic transcription factor involved in cell-cycle regulation, proliferation, growth, metabolism and differentiation (Schmidt, 1999). It belongs to the oncogenes, and when over expressed, it often causes cancer. c-myc is involved in both self-renewal and differentiation of stem cells and progenitor cells. Murphy et al. found several c-myc binding sites throughout the genome indicating a possible function of this gene in recruitment of chromatin-remodelling activities to promoters (Murphy et al., 2005). C-myc is dispensable for reprogramming somatic cells to a pluripotent state and can be replaced with other members of its family. But the reprogramming efficiency decreases when c-myc is omitted (Eminli et al., 2008; Huangfu et al., 2008b; Kim et al., 2008; Nakagawa et al., 2008; Wernig et al., 2008).

Even though reprogramming with the four Yamanaka factors seems to be a very effective way for generating iPS cells, in view of a possible therapeutic application this leads to concerns regarding the safety of this methodology. An important goal of the researchers in this field is and will be in the next future to optimize the methodology in terms of safety and efficiency.

5.2. Alternative methods to generate iPS cells

The need for alternative methods to generate iPS cells comes from the fact that c-myc and Klf4 are oncogenes (Meyer and Penn, 2008; Rowland and Peeper, 2006). But the safety issues of iPS cells are not only caused by the use of c-myc and/or Klf4 for reprogramming, particularly because c-myc is dispensable for reprogramming (Wernig et al., 2008). The use of retro-or lentivirus for mediating the transduction of reprogramming causes safety issues. Retrovirus and lentivirus, both integrate into the genomic DNA of the host cell (Fig. 7). The insertion of viral sequences into DNA might cause muta-

tions. Hence, many laboratories tried to minimize the use of integrating viruses. As a first approach some studies took NS cells or neural progenitor (NP) cells to generate iPS cells. The advantage of these cells is that they already express Sox2 endogenously and therefore can be reprogrammed only with the two factors Oct-3/4 and Klf4 (Eminli et al., 2008; Kim et al., 2008). Besides that, generation of iPS from adult mouse NS cells by using only Oct-3/4 has been reported. In all probability it was possible because of the endogenous expression of Sox2 and Klf4 in NS cells (Kim et al., 2009b).
Another possibility is to express the four factors by a single viral vector. Therefore one has to use the combination of the IRES and 2A peptide sequence technology (Carey et al., 2009; Sommer et al., 2009).
By using NS cells or NP cells or the 2A peptide/IRES (internal ribosomal entry site) technology for reprogramming, the number of viruses can be reduced, thus the possibility of mutations is minimized. In 2008, Silva et al. reprogrammed mouse brain-derived NS cells and could show, that the reprogramming procedure was faster than for other cell types. But once reprogrammed, critical attributes of pluripotency such as stable expression of endogenous Nanog and Oct-3/4, epigenetic erasure of X chromosome silencing in female cells and the ability to colonise chimeras, could not be obtained in these cells. Therefore, they applied molecularly defined conditions designed to perpetuate ES cells in a pluripotent ground state by neutralizing inductive differentiation stimuli (Silva and Smith, 2008; Ying et al., 2008). More precisely, they cultivated ES cells under serum-free conditions with LIF and two small molecule inhibitors which block the pathway of Mek (mitogen activated protein kinase)/Erk and the GSK3 pathway. With the 2i/LIF conditions the authors succeeded to generate iPS cells from mouse brain-derived neural stem cells, which had a stable up-regulation of Nanog and Oct-3/4, a reactivated X chromosome and were able to contribute to germline chimeras. Besides that, they could demonstrate, that the 2i/LIF conditions allow generating iPS cells only with two factors (Klf4 and Oct-3/4) (Silva et al., 2008). Under these conditions, NS cells reprogram faster paired with a high incidence of conversion. Their approach might lead, in general, to the establishment of new protocols to reprogram faster and for a more stable cultivation of reprogrammed cells.
Other groups demonstrated that other small molecules could replace reprogramming factors and increase efficiency. The use of BIX-01294, an inhibitor of the G9a histone methyltransferase, in NP cells circumvents the necessity of Oct-3/4 for reprogramming. In presence of Oct-3/4 and Klf4, BIX-01294 promotes the reprogramming efficiency (Shi et al., 2008b). Shi et al. were able to show, that a combination of BIX-01294 and BayK8644 (a L-channel calcium agonist) allows the reprogramming of MEFs with only two factors (Oct-3/4,Klf4)(Shi et al., 2008a). Another research group described the generation of iPS cells from human fibroblast by introducing Oct-3/4, Sox2 together with valporic acid (VPA), which acts as a histone deacetylase inhibitor (Huangfu et al., 2008b). In both, human and mouse cells, VPA increases the efficiency of reprogramming dramatically, whether they are infected with two, three or four factors (Huangfu et al., 2008a). The use of VPA together with Oct-3/4 and Sox2 allows the substitution of c-myc and Klf4. Taken together, the findings of Shi et al. and Huangfu

et al., highlight the involvement of chromatin remodelling in the process of reprogramming, the exact process is nevertheless still undefined. However, the discovered increasing effect of VPA for reprogramming can be used for strategies, which have to deal with low reprogramming efficiency. Stadtfeld et al. succeeded in 2008 to use adenoviral vectors instead of retroviral ones, to bring mouse hepatocytes to an embryonic state (Stadtfeld et al., 2008). The advantages of the adenoviral approach are, that this virus does not integrate into the host genome as part of their life cycle (Fig. 7).
But still, the efficiency of retroviral delivery is much higher, compared to the repeated adenoviral infection. In addition, the use of adenovirus often leads to tetraploidy in the resulting iPS cells. In the same year another group published their data and described the generation of iPS cells by plasmid transfection (Fig. 7) (Okita et al., 2008). As with adenoviral approach, efficiency decreased compared to retroviral expression.

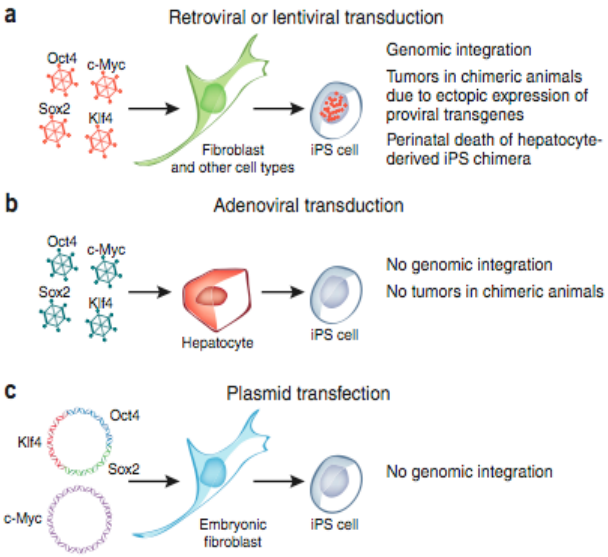


Fig 7: Different strategies to generate mouse iPS cells.
In approach **a**, the viral vector is integrated into the host genome
In contrary, in approach **b** and **c** no integration occurs (from Lowry, 2008).

One method to avoid insertion mutations is to reprogram somatic cells with piggyBac (PB) transposon. By using PB, the integrated exogenous genes can be removed by transient expression of transposase in the resulting iPS cells. These iPS cells seem to be free of PB transposon integration sites. Nevertheless, if the PB transposon insertions while iPS formation have any long-term effect, remains to be determined (Chen and Liu, 2009). The most auspicious method, relating to the safety of iPS cells, to bring somatic cells to a pluripotent state is not to introduce genes, coding the required factors, but to deliver the reprogramming proteins. By the utilization of cell-penetrating peptides, the reprogramming proteins can be brought into the cells. So far, protein-induced iPS cells could be established from mouse and human cells (Kim et al., 2009a; Zhou et al., 2009). Even if the efficiency is reduced, this

approach is so far the safest method of generating iPS cells in perspective of clinical applications. In summary, all the alternative strategies have to be improved, in order to increase reprogramming efficiency. The identification of new small-molecules (e.g. DNA methyltransferase inhibitors, histone deacetylase inhibitors, inhibitors of Mek and GSK3 pathways) together with the identification of more appropriate cells to induce iPS cells (such as keratinocytes and adult stem cells) could further enhance the iPS generation efficiency (Aasen et al., 2008).

6. Aim of the work

The rat, compared to the mouse, offers as a model for human diseases many advantages. Since it was once the most widely used organism in medical research, a lot of data has already been generated, and in addition, the physiology of the rat is easier to monitor and is more similar to the human organism one.

For many years the derivation of rat ES cells was impossible and only recently it was for the first time described the generation of rat embryonic stem cells able to generate a chimeric animal. Nevertheless, at the moment no reports are existing confirming the use of rat ES cells line for gene targeting. The main problem is that only a very stable ES cell line is able to maintain its germline competence after the long and tedious process of gene targeting. The discovery of iPS cells could substantially improve the development of new gene targeting approaches in the rat and making the use of ES cells dispensable.

Because the development of the iPS technology is based on mouse ES cells research and mouse strains variations strongly influences the derivation of ES cells it was interestingly to examine if it is possible to generate rat iPS cells from different rat strains.

All the reprogramming experiments will be performed by using only three factors (Oct-3/4,Sox2, Klf4).

The following experiments are planed:

- reprogramming of inbred Brown Norway rat embryonic fibroblast.
- reprogramming F1 crossing between Sprague Dawley and Brown Norway rat embryonic fibroblasts.
- reprogramming F1 crossing between Sprague Dawley and Brown Norway rat embryonic fibroblasts carrying a knockout alle for the neurotrypsin gene

In the second part of the work, the influence of leukemia inhibitory factor on reprogramming will be analyzed. The recently discovered gene Pramel7 was shown to play an important in conferring pluripotency to embryonic stem cells. Its overexpression is sufficient to maintain pluripotency in ES cell in the absence of LIF. Previous experiments indicate that Pramel7 could be the effector gene of the LIF/Stat3 pathway. The presence of LIF in the reprogramming medium is very important, and LIF is considered to be necessary for the correct reprogramming of the fibroblasts. Normally the presence of LIF is guarantied through the presence of a SNL feeder layer consisting of cells that overexpress LIF.

By using a conditionally active form of Pramel7 the following experiment are planed in order to analyse if Pramel7 has an influence in the reprogramming efficiency of mouse embryonic fibroblasts and if its overexpression is sufficient to reprogram the cells without the presence of LIF.

All the reprogramming experiments will be performed by using only three factors (Oct-3/4,Sox2,Klf4).

The following experiments are planed:

- reprogramming of wild type mouse embryonic fibroblasts under the following four culture conditions:

1. iPS-medium + LIF
2. iPS-medium + OHT
3. N2B27 + 2i + OHT
4. N2B27 + 2i + OHT + LIF

- reprogramming of transgenic mouse embryonic fibroblasts carrying the Pramel7_MER construct under the following culture conditions:

1. iPS-medium + LIF
2. iPS-medium + LIF + OHT
3. iPS-medium + OHT
4. N2B27 + 2i + OHT
5. N2B27 + 2i + OHT + LIF

B. Materials

1. Chemicals, biochemicals and enzymes

Trypsin-EDTA solution 10x, SIGMA
Mitomycin C, SIGMA
Dulbecco's phosphate buffered saline, SIGMA
Fugene 6, Transfection Reagent, Roche
Polybrene Infection / Transfection Reagent, Millipore
4% Paraformaldehyde, Fluka
4-Nitro blue tetrazolium chloride, Roche
5-Bromo-4-chloro-3-indolyl-phosphate, X-phosphate, 760 994, Roche
Accutase Enzymes, Sigma

2. Definitions and preliminary remarks

The methods are generally described

In the following pages the term fibroblasts is always used to define embryonic fibroblasts that are not mitotically inactivated. As feeders were defined fibroblasts which were inactivated with mitomycin C.

All media were stored at 4°C and preheated to 37°C before use.

3. Cells and cell culture media

3.1. Platinum-E (Plat-E) cells

Derived from 293T cell line, Plat-E cells contain an env-IRES-puro cassette and a gag-pol-IRES-cassette. They were cultivated with the same medium used for fibroblast culture (see below).

3.2. Rat fibroblast

Wild type fibroblasts: Derived from Brown Norway rat strain and from the SDxBN hybrid.

Transgenic fibroblast: SDxBN carrying Neurotrypsin gene knock-out.

Fibroblast medium (rFb-medium)

D-MEM 1x, GIBCO

10% Fetal Bovine Serum, heat-inactivated, GIBCO

Penicillin, Streptomycin (10.000 U/ml penicillin G sodium, 10.000µg/ml streptomycin in 0.9% NaCl), Sigma

Sodium Pyruvate 100mM (11.0mg/ml sodium pyruvate in tissue culture grade water), GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

Freezing medium for fibroblast

rFb medium cont. 10% DMSO, Sigma

3.3. Mouse fibroblast

SNL fibroblasts available from Dr. Allan Bradley of the Sanger Institute, derived from a STO cell line. SNL 76/7 STO cells contain a neomycin-resistant cassette and express leukemia inhibitory factor (LIF).

CD-1 fibroblasts derived in LTK Institute

SNL-medium

D-MEM 1x, GIBCO

7% Fetal Bovine Serum, heat-inactivated, GIBCO

L-Glutamin, Penicillin, Streptomycin (10.000 U/ml penicillin G sodium; 10.000µg/ml Streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl), GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

3.4. riPS cells

iPS-medium

D-MEM 1x, GIBCO

15% Fetal Bovine Serum, heat-inactivated, GIBCO

L-Glutamin, Penicillin, Streptomycin (10.000 U/ml penicillin G sodium; 10.000µg/ml streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl), GIBCO

Sodium pyruvate 100mM, SIGMA

ß-Mercaptoethanol 50 mM, GIBCO

MEM Non Essential Amino Acids 100X, GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

Supplemented with: 10^7U/ml, 10.000X ESGRO murine LIF, Chemicon Int.

N2B27 medium

DMEM/F-12, Invitrogen

Neurobasal medium, Invitrogen

2-Mercaptoethanol 50 mM, GIBCO

Glutamine 100X, Invitrogen

B27 50x, Invitrogen

N2 100x, Invitrogen

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

Supplemented with: 10^7U/ml, 10.000X ESGRO murine LIF, Chemicon Int.
3µM CHIR99021, STEMGENT
1µM PD0325901, STEMGENT

iPS cells freezing medium

Fetal Bovine Serum, heat-inactivated, GIBCO

10% DMSO (Dimethyl sulphoxide), SIGMA®

4. Culture dishes

24/96 Multidishes, Corning

35/60/100mm plates, Corning

150mm plates, TPP

5. Material

15ml/50ml tubes, GREINER

CryoTube™ vials, Nunc™

Filter bottles (0.2µm pores), Corning

Serological pipettes, Sarstedt or Falcon

20ml syringe, Once

0.45µm syringe filter, Whatman

Dissection scissor, Aesculap

6. Equipment

Centrifuge (Megafuge 2. ORS), Heraeus

Centrifuge 5810R, Eppendorf

Centrifuge 5418, Eppendorf

Minifuge 5415R, Eppendorf

Thermo mixer, Eppendorf

Incubator Cytoperm2, Heraeus

Microscope: Nikon TMS / Nikon Eclipse E600 / Nikon SMZ 800

Sterile bench: VSE 2000-120, SKAN

7. Software

AxioVision Rel. 4.6

Endnote X1

C. Methods

1. Rat feeders / SNL feeders / CD-1 feeders

Mitotically inactivated fibroblasts are used as basal layer for iPS cells and secret factors, provide extracellular matrix and cellular contact to maintain the cells in undifferentiated and pluripotent state.

1.1. Production of Rat fibroblast

Rat fibroblasts were derived from either embryonic day 13.5, 14.5 or 15.5 (E13.5, E14.5, E15.5) foetuses. Pubs were removed from uterus, released from the amniotic sac. Heart, liver and head were removed and were fragmented to smallest possible pieces using a dissection scissor. Resulting tissue fragments were then further homogenised using 1x trypsin.

Finally the cells were resuspended in rFb-medium and transferred to 150mm dishes ($5x10^6$ cells/150mm). They were incubated at 37°C, 5% CO_2 in air and 95% relative humidity (37°C, 5% CO_2, 95% rH) until the cells became confluent. At this point the cells were either expanded or frozen as passage 0.

1.2 Expansion of Rat fibroblast

Rat feeders are quite difficult to expand, because they almost do not adhere to the dish. Due to this the fibroblasts were plated in 1 gelatinized 15 cm plate instead of 4 gelatinized 15cm plates after thawing. Furthermore they were only expanded as far as passage 1.

1.3 Expansion of SNL-fibroblasts

At day 1 a vial of SNL-feeders was thawed and plated in four 15cm plates, using 20ml of SNL-medium for each plate. During the next 5 days the cells were cultivated without changing the medium. At day 6 the plates were confluent and the cells were mitotically inactivated.

1.4 Inactivation of Rat fibroblasts

Rat fibroblasts were inactivated at passage 1. The inactivation is performed with mitomycin-C, a cytostatic antibiotic. The medium was removed from the 10cm culture dishes and 3.5 ml of mitomycin-C was added to each plate. Then the plates were incubated for 2hs at 37°C. After the incubation time, the mitomycin was aspirated and the cells were washed three times with 10ml PBS. Afterwards 3ml 1xtrypsin was added to the cells and then incubated for 3min at 37°C. The 1xtrypsin was stopped with 7ml Fibro medium and the cells resuspended. Aliquots of 10µl were taken to determine the amount of cells. The suspension was centrifuged for 4min at 210g. The pellet was resuspended in 6ml freezing medium and aliquoted in 6 cryotubes containing 2.4 million cells each, or the cells were directly plated either on a 10cm dish or 6cm dish for the reprogramming. When resuspended in freezing medium, the tubes were stored over night at -80°C and then transferred in the liquid nitrogen tank.

2. Reprogramming

Day 1: Preparation of PlatE-packaging cell: During the experiments, 5 10cm and 15 6cm dishes with rat embryonic or mouse embryonic fibroblasts were reprogrammed, meaning that totally 117.5ml (5x10ml and 15x4.5ml) of retrovirus medium containing the three factors (SOX2, Oct-3/4, Klf4) were needed. To produce the medium, 15 vials of PlatE cells (1/2 10cm) were thawed, transferred on 15 10cm gelatinized cell culture dishes and incubated in Fibro medium over night at 37°C, 5% CO_2, 95% rH until confluence.

Day 2: Transfection of pMXs vectors into PlatE packaging cells: In total 15 10cm dishes with PlatE cells were transfect. For the first reprogramming round 3 10cm dishes with PlatE cells were transfect, so it was for the second and third round. In the fourth round 6 10cm dishes were transfect. Here I describe the transfection of 3 10cm dishes with PlatE cells. For 6 dishes the same protocol was used.

For transfection, 9µg of plasmid DNA (SOX2, Oct-3/4, Klf4) were needed fro 1 10cm dish. According amounts were pipetted into 3 1.5ml tubes. Then 3 other tubes were prepared, each containing 300µl of DMEM. After drop wise addition of 27µl Fugene6 reagent to the DMEM, the solution was incubated fro 5' at room temperature. During this incubation step, the medium of PlatE-plates was exchanged (Fibro medium). Then pMXs vectors were added drop wise to the Fugene/DMEM solution, ending up with 3 tubes containing DMEM, a pMXs vector and transfection reagent. After 15' of incubation at room temperature, app. 310µl of plasmid/transfection reagent solution were added to a single PlatE plate, resulting in1 plate per vector (SOX2, Oct-3/4, Klf4). PlatE plates were incubated over night at 37°C, 5% CO_2, 95% rH.

Day 3: Preparation of SNL/Rat/CD1-feeders:

According to the experiment, SNL and Rat feeders were used to reprogram Rat embryonic fibroblast (REFs) and CD1-feeders were used to reprogram Mouse embryonic fibroblasts (MEFs).

In the morning PlatE cells were supplied with fresh Fibro medium. Then SNL or CD1-feeders were prepared in order to have confluent plates for the next day. The Rat feeders were already confluent inactivated, so that they could be used the next day. In total, 4 10cm plates with SNL feeders (app. $3x10^6$cells/plate), 14 6cm plates with CD-1-feeders (app. $7.2x10^4$cells/plate), 1 10cm and 1 6cm plate with Rat feeders (undefined amount of cells, but confluent) were used. The plates were incubated at 37°C, 5% CO_2, 95% rH until the next day.

Day 4: Transfection of MEFs/REFs:

Retrovirus containing medium of PlatE plates was aspirated by using a 20 ml syringe and afterwards transferred into a 50ml tube by pushing the solution through a 0.45µm syringe filter. The three different media were always pooled in 1 50ml tube. When retrovirus containing medium was collected from 6 PlatE plates, it resulted in 2 50ml tubes containing 30ml (10ml for every plasmid) retrovirus medium, when collected from only three PlatE plates 1 50 ml tube containing 30ml pooled retrovirus medium was received. Thereafter, 15µl Polybrene solution was added to 30ml pooled medium. Afterwards,

medium of SNL/CD1/Rat-feeders was exchanged with 10ml of virus medium for a 10cm dish and 4.5 ml of virus medium for a 6cm dish. As a last step, 1ml of MEFs or REFs solution containing either 800'000 cells fro a 10cm reprogramming plate or 400'000 for a 6cm plate was added to the virus medium containing feeder plates.

Addition: For the reprogramming of the neurotrypsin knock out fibroblast, in a first reprogramming round only 97'500 cells were thawed and used for reprogramming in 10cm dish. In second round, 175'000 cells were reprogrammed in a 6cm dish.

Days 5-6 (days 1-2 post transfection (pt)): Fibro medium was changed.

Day 7: REFs → Fibro medium was replaced by iPS medium.

MEFs → 7 different media were used to replace the Fibro medium.

For Pramel7_MER and STAT3_MER murine fibroblasts 5 different media were used:

iPS medium + LIF + OHT

iPS medium + LIF

N2B27 medium + 2i + LIF + OHT

N2B27 medium + 2i + OHT

iPS medium + OHT

For WT murine fibroblasts 4 different media were used:

iPS medium + LIF

N2B27 medium + 2i + LIF

N2B27 medium + 2i

iPS medium + OHT

Days 8-9 (days 4-5 pt):

In every reprogramming approach, media were changed every- or every second day. Morphological changes were observed every day. In the different experiments the resulting iPS colonies showed their typical ES cell-like morphology including round and shiny borders at different days pt, thus were also on different days pt ready for picking.

For wild type REFs reprogrammed on a SNL feeder layer: at day 14-15 pt.

For transgene REFs reprogrammed on a SNL feeder layer: at day 35 pt.

For wild type REFs reprogrammed on a Rat feeder layer: at day 13 pt.

For transgene REFs reprogrammed on a Rat feeder layer: at day 14 pt.

iPS-colonies from MEFs were directly stained and therefore not picked

2.1 Picking and expansion of iPS-colonies

iPs-colonies from the total three performed reprogramming rounds were picked at different days pt (see above). Therefore a 96 well plate was prepared with either 30µl of PBS or 50µl of Accutase per well and picked colony. Colonies were picked with 10µl pipette and flushed into prepared wells. After picking, 30µl of 2x Trypsin was added each well, when colonies were picked in PBS and afterwards incubated for 5' or 10' at 37°C, 5% CO_2, 95% rH. If colonies were picked in Accutase, they were after

being picked, incubated for 1/2h at 37°C, 5% CO_2, 95% rH. After incubation, 2x Trypsin and Accutase were stopped with 100µl iPS-medium + LIF. In a next step, the colonies were resuspended by intensively pipetting up and down and then transferred into 24 well plate that were previously covered with SNL/Rat-feeders and supplemented with iPS-medium + LIF.
The following days colony growth was observed. In order to bring the colonies to confluence, they were split 1:1 from 24 well into 24 well/3.5cm culture dishes or from 3.5cm to 6cm culture dishes. Some iPS cells were frozen from 3.5 cm dishes with freezing medium.

2.2 Analysis of iPS cells

The following characterizations approaches were applied:

2.2.1 Check of marker of pluripotency: Alkaline Phosphatase and IHC

As a first step, medium was removed from 6cm culture dishes. After a washing step with PBS, 1ml of 0.4% Formalin was added fro 15' to each dish to fix the cells at room temperature. Then iPS colonies were washed 3 times with PBS, 10'each step. At this point, cells could be stored in PBS at 4°C until they were used for staining.

2.2.2 Alkaline Phosphatase (AP)

After the cell fixation they were washed twice 10min with "AP-buffer" (100mM TrisCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$). The development was performed for 2-3 hours in AP-buffer containing 0.5 µl/ml NBT (75 mg/ml in 70% dimethylformamide) and 3.5 µl/ml BCIP (50mg/ml in 100% dimethylformamide). After the incubation time the AP expression was check: pluripotent cells have a higher AP expression than differentiated cells, and therefore they appear blue. Differentiated colonies appear white as well as feeders. The cells were washed once with PBS and then the staining reaction was blocked with Tris EDTA. The cells were incubated for 10min at room temperature in this solution. In order to eliminate precipitates they were then washed 3 times with PBST (PBS cont. 1% of Tween) shaking at 40rpm. Pictures of the colonies were taken.

2.2.3 Immunohistochemistry (IHC)

For antibody staining, cells firstly had to be washed 1x with PBST (PBS cont. 1% of Tween) for 10'. Then primary antibodies, diluted in 0.5 ml PBST and 4% horse serum, were added. After incubation o.n at 4°C, antibody solution was removed and cells were washed 3x 10' with PBST followed by a washing step with PBS (10'). Secondary antibodies in 0.5ml of PBS were added for 2-4 h at room temperature. Incubation had to occur in the dark due to loss of fluorescence intensity of secondary antibodies when exposed to light. After removal of antibody solution and 3x washing with PBS, cells were stored in 2ml of PBS for further analysis.

Primary antibody (dilution)	Secondary antibody (dilution)
Rabbit α Oct-3/4 (200µg/ml, Santa Cruz Biotechnology) 1:500	Alexa Fluor 594 goat α rabbit IgG (2ml/mg, Invitrogen) 1:500
Mouse α SSEA-1 (1mg/ml, Chemicon Int.) 1:200	Alexa Fluor 594 goat α rabbit IgG (2mg/ml, Invitrogen) 1:500

Tab. 1: Table of antibodies used for Immunohistochemistry

3. Reverse transcription and quantitative Real-Time PCR (RTQ-PCR)

Cells were first washed with PBS, then covered with 1x trypsine and incubated for 5' at 37°C, 5% CO_2 and 95% rH. After 5', separation of cells was checked. After detachment of all cells, trypsine was stopped by adding iPS medium. Afterwards, the solution was centrifuge for 4' at 210g. Pellet was resuspended in 350µl RLT-buffer (Qiagen) cont. 1% 2-mercaptoethanol (Fluka), transferred in a 1.5ml tube and stored at -80°C.

RNA was extracted by using Qiashredder® and RNeasy® kits (both Qiagen). Concentration of RNA was measured with NanoDrop® and the RNA quality was determined by electrophoresis analysis, using a Bioanalyzer® (Agilent). 0.5µg of RNA were reverse transcribed using oligo-dt primers (Invitrogen) and Superscriptlll (Invitrogen).

Real-Time PCR (RT-PCR) was carried out by using the QuantiTect SYBR Green PCR Kit (Qiagen) and a Rotor-Gene 6000 real-time-thermocycler (Corbett). Per RT-PCR reaction the total volume was 20µl: 18µl of master mix plus 2µl of template.

Quantitative RT-PCR for each gene was done in triplicates and the values were normalized to the corresponding amounts of β-actin.

The RT-PCR parameters were the following:

Initial denaturation: 95C° : 15min

Denaturation: 94°C : 15s

Annealing: 58°C : 30s

Elongation: 72°C : 30s (acquiring to Cycling A (Green))

→ Number of cycles: 40

Primer	**Sequence**
Pramel7 backward	5'-CCA AAG CCA TGT GGG TTC TC-3'
Pramel 7 forward	5'-GGT GAC AGA TGC CTA CTA TGA G-3'
β-actin backward	5’-GAT CTT CAT GGT GCT AGG AGC CAG AGC-3’
β-actin forward	5’-CAT CCA GGC TGT GCT GTC CCT GTA TGC-3’

Tab. 2: List of primers for Quantitative Real -Time-PCR

D. Results

1. Reprogramming of rat fibroblast from different strains

In total two reprogramming rounds were performed with only three reprogramming factors (Oct-3/4, Klf4, Sox2) - c-myc was omitted because of its cancerogenous properties. For reprogramming fibroblasts from different rat strains were used.

The fibroblasts used for the first round of reprogramming were derived from embryos (day 14) obtained from a Sprague Dawley x Brown Norway (SDxBN) crossing. Oliver Sterthaus, previously used these fibroblasts during his PhD-thesis at the Institute of Laboratory Animal Science, to perform gene-targeting experiments aimed at the deletion of the neurotrypsin gene (an approach towards gene-targeting in rat using the nuclear transfer technology). Both the homologously recombined fibroblasts (KO fibroblasts) and the original WT fibroblasts were reprogrammed during this master thesis. In the second round of reprogramming, fibroblasts derived from the Brown Norway (BN) rat strain were used.

For all experiments with WT cells 800 000 fibroblast of each strain were used and reprogramming was performed in a 10 cm cell culture dish. Due to the small amount of KO fibroblasts available for the reprogramming experiments, only 175 000 cells were used. The first round of reprogramming was performed in a 10 cm dish and the second in a 6 cm dish.

The aim of the reprogramming procedure was to generate stable iPS colonies that could be further expanded as clones for immunohistochemical analysis with pluripotency markers and for storage in liquid nitrogen for future blastocysts injections and gene targeting experiments

1.1. Reprogramming of wild type and knock out SDxBN fibroblasts

In the first reprogramming round, WT fibroblasts and KO fibroblasts of the SDxBN strain were reprogrammed. Both the reprogramming and the expansion of the cells were performed by using SNL-feeder-layers that overexpress LIF.

iPS colonies derived from WT fibroblasts were ready for picking at infection day 15 (id 15), whereas the KO cells showed no colonies at this point of reprogramming (Fig. 1). The KO cells were kept in culture and the medium was changed every second day.

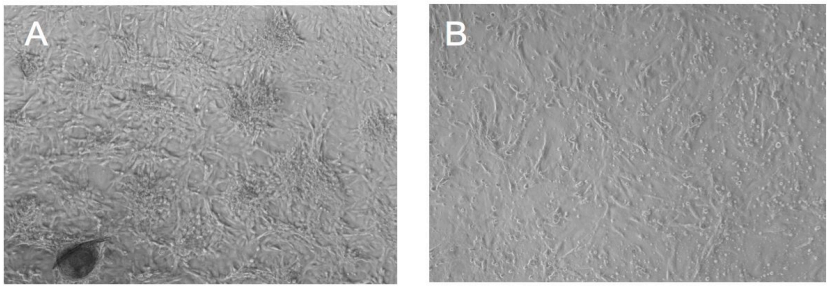


Fig. 1: Colony formation at id 15. **A:** Formation of colonies form the reprogrammed WT REFs. **B:** No colony growth from the reprogrammed KO REFs. Magnification: 5x

1.1.1. Expansion of SDxBN wild type iPS cells

At id 15, 24 WT iPS colonies were picked and single colonies were transferred into a 24 well dish. The reprogramming efficiency of the WT fibroblasts was very high and 24 more colonies were further picked at id 19.

5 days after picking new colonies were observed in the 24 wells. In order to increase the number of colonies, cells were split at a ratio 1:1 into a new 24 well plate coated with SNL feeders and the medium was changed every second day.

As already observed in previous experiments the formation of new ES cell-like colonies after splitting of the iPS cells can take between 1-3 weeks. This could be observed with the WT SDxBN iPS cells, but in contrast to previous experiments, the number of colonies was not increased as expected. No clone was obtained, which formed more than 5 colonies after dissociation of the original colony. These few colonies were very dense and compact and, with the time, were turning darker, probably because the cells were becoming necrotic (Fig. 2). In order to increase the number of colonies and prevent their loss because of cell death, the 48 clones were further split at a ratio 1:1 into 3.5 cm cell culture dishes.

Fig. 2: WT riPS cells: WT riPS cells in the 24 well dish before splitting into a 3.5 cm dish. The colonies are very dense and turning black. Magnification: 10x

Dissociation of the cells was performed by incubating the cells for 10 minutes with trypsin. The dissociation process was checked under the microscope till a single cell suspension was obtained. Two days after the last passaging, the single clones showed different growth behaviours: only 8 out of 48 clones showed an increased number of colonies, in other 20 dishes only 1-7 colonies per clone were visible and in the remaining 20 dishes no colonies were present (Tab. 1).

Growth behaviour	Fast (> 7 colonies)	Slow (1-7 colonies)	no growth (no colonies)
Clone number	C 5	C 3 C 29	C 1 C 41
	C 7	C 4 C 30	C 2 C 42
	C 14	C 8 C 32	C 6 C 44
	C 23	C 10 C 38	C 9 C 45
	C 34	C 11 C 40	C 13 C 47
	C 35	C 12 C 46	C 15 C 48
	C 36	C 16	C 19
	C 43	C 17	C 26
		C 18	C 27
		C 20	C 28
		C 21	C 31
		C 22	C 33
		C 24	C 37
		C 25	C 39

Tab.1: Growth behaviour of the different clones

At this point only the 8 “fast growing” clones were further expanded (Fig. 3). The clones, which showed 1-7 colonies, were frozen and stored in the liquid nitrogen tank and all the other clones, which did not grow, were eliminated.

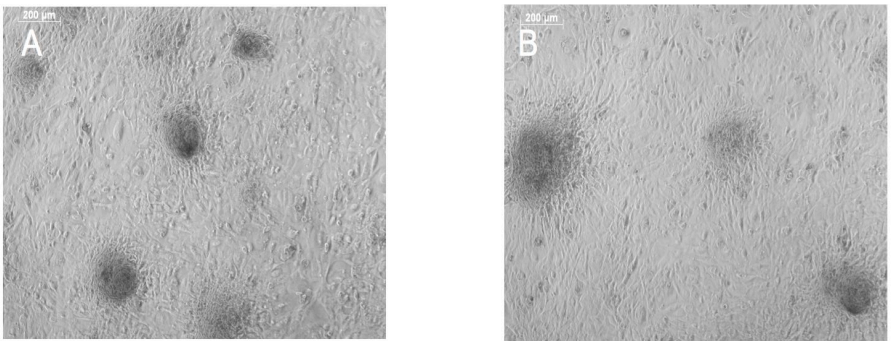


Fig. 3: Clone 4 **(A)** and Clone 7 **(B)**, 1 day after the transfer from 24 well to the 3.5 cm dish. Magnification: 5x

The 8 “fast growing” clones were split four times every fourth day in order to expand the colonies. Instead of an increasing a decrease in the number of colonies was observed. These iPS cell clones never showed a normal proliferating behaviour, the colonies were very small and compact (Fig. 4) and did not grow anymore. Because no further expansion could be obtained, after 4 weeks (passage 8), all the remaining plates had to be eliminated.

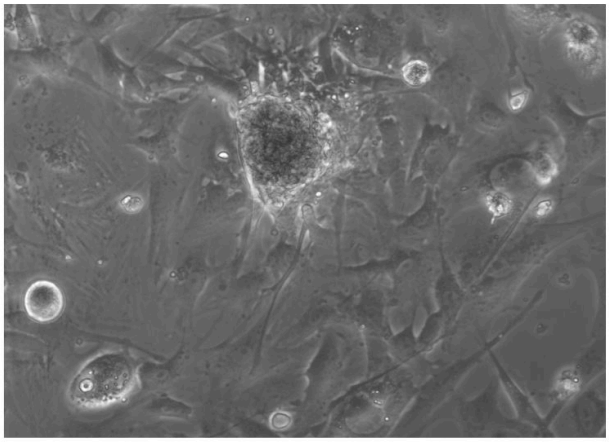


Fig. 4: SDxBN iPS colony at passage 7. The colony is small and compact in its morphology. Magnification: 10x

1.1.2. Expansion of knock out iPS-cells

At id 34, ES cell-like colonies were observed in the reprogramming plate of the KO fibroblasts. Interestingly all the colonies grew on the border of the dish (hence no pictures could be taken).

48 colonies were picked and cultivated in 24 well plates on SNL feeders. 4 days after picking, in each well only one colony was observed, meaning that the trypsinisation step has not been successful. Therefore all the 48 wells were split at a ratio 1:1 into new wells. 7 days later, only in 3 wells one colony could be seen. These colonies did not growth anymore and the cells died.

Because in the original reprogramming plate colonies were still present, the complete plate was trypsinised and the cells distributed into 8 wells of a 24 wells plate. In each of these 8 wells clones with different growing characteristics could be observed. 5 days after the trypsinisation of the original plate, each of the 8 wells were split into 8x 3.5 cm dishes in order to increase the amount of colonies. This time, the cells were incubated for 30 min. with accutase instead of trypsin, which is not toxic for the cells, also when incubated for a long time. After this treatment, no expansion of colonies could be seen. Therefore, 3 days later, the cells were split again at a ratio 1:1 in 8 new 3.5 cm dishes by incubating them with Accutase for 45 min. Also after this splitting step, no colony expansion could be noticed.

In order to check, if the splitting steps have an influence on the number of colonies, the colonies from each 3.5 cm dish were counted before and after the next splitting step with Accutase (Tab. 2). This time the cells were not transferred into new 3.5 cm dishes, in order to avoid losing colonies while transferring them.

Plate number	1	2	3	4	5	6	7	8
Number of colonies before splitting	6	1	7	2	12	3	5	0
Number of colonies after splitting	5	1	6	2	12	3	2	0

Tab. 2: Comparison of the amount of colonies, before and after the third splitting step. For all 8 plates, no increased number of colonies could be obtained.

In the next few days no colony growth was observed and the remaining colonies became small and strange in morphology (Fig. 5). Therefore, 5 days after the last splitting, plate number 2 and 8 were discarded and the rest of the plates were then transferred again in new 3.5 cm dishes coated with rat-feeders, but the cells died.

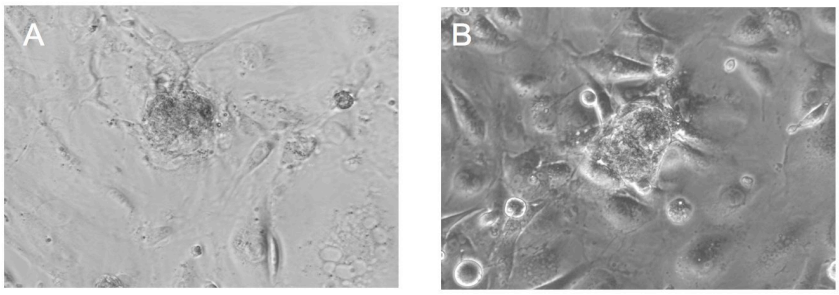


Fig. 5: After 3 splitting steps. Colonies became small and showed a strange morphology.
A: Plate 5 **B:** Plate 3. Magnification: 10x

In this first reprogramming round no clone could be expanded. Therefore, the reprogramming was repeated by changing some cell culture conditions.

1.2. Reprogramming of Brown Norway and KO (BNxSD) fibroblasts

In the second reprogramming round were used WT fibroblasts derived from BN rats and KO fibroblasts from a F1 breeding between Brown Norway and Sprague Dawley rats (the same as in the first round). This time, dishes coated with rat feeders instead of SNL feeders were used for the reprogramming as well as for the expansion until passage 1.

At id 10, the first colonies derived from WT fibroblast were visible. In the reprogramming plate from the KO fibroblast, the first colonies appeared at id 12. By using a rat-feeder layer, colony formation was faster compared to the first reprogramming round and was in accordance with previous observations. Especially, ES cell-like colonies derived from the reprogrammed KO fibroblasts appeared after 12 days, means 22 days before their appearance in the first round.

13 days after the infection, WT fibroblasts were ready for picking, whereas the colonies obtained from the KO fibroblasts were picked at id 14 (Fig. 6).

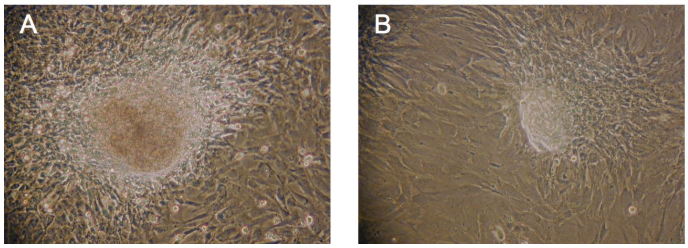


Fig. 6: A: BN-iPS cells at the day of picking (id 13). **B:** KO iPS cells at the day of picking (id 14). Magnification: 20x

1.2.1. Expansion of BN iPS cells

24 WT BN iPS colonies were picked at id 13 and seeded into a 24 well dish. Another 24 colonies were picked at id 15. Only in 4 wells a single colony was visible, indicating that probably the dissociation with trypsin did not work properly. In the other wells no colonies were visible, this could indicate that dissociation was performed correctly or that the colonies were lost during transfer to the 24 well plate. 5-7 days after picking, in some wells new colony growth could be observed. 12-14 days after picking 11 wells were nearly confluent and the cells were therefore transferred into 3.5 cm dishes coated with SNL feeders (Fig. 7).

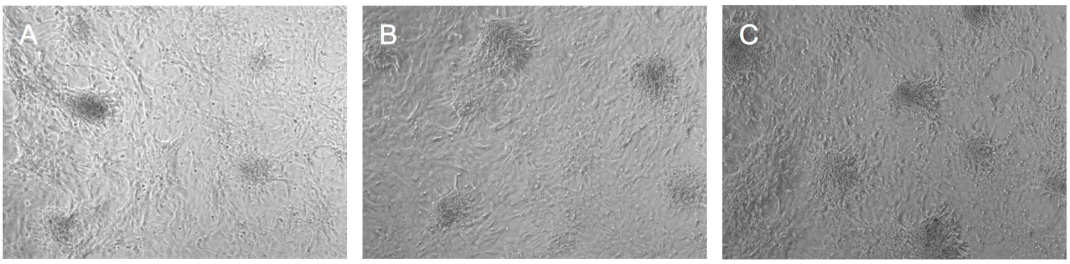


Fig. 7: Growing BN iPS colonies. **A:** 6 days after picking. **B:** 8 days after picking. **C:** 12 days after picking. Magnification: 5x

For the next 10 days the medium was changed every second day in the eleven 3.5 cm dishes and colony growth was checked every day under the microscope. It took 10 days before new colony formation could be observed in a first dish. Another 5 days later, in total 4 clones showed growth of new colonies and pictures were taken (Fig. 8).

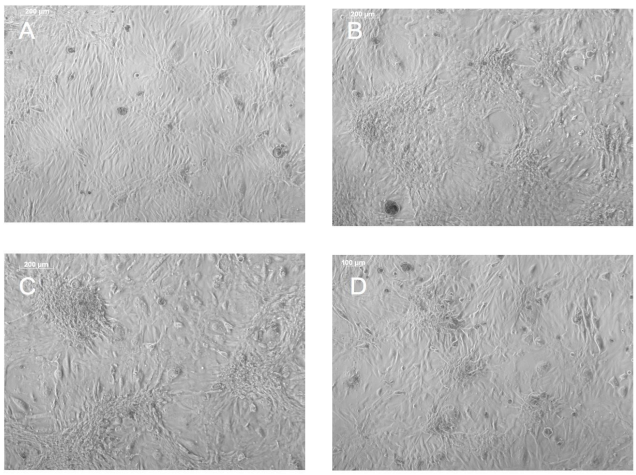


Fig. 8: A-D: The 4 first clones which showed new colony formation after being transferred into a 3.5 cm dish. Magnification: 5x

These 4 clones were then split the next day at a ratio 1:2 into new 3.5 cm dishes in order to have two dishes from each of the 4 clones to have one plate for staining with markers of pluripotency and one plate to freeze down and store.

1.2.1.1. Analysis by immunohistochemistry and alkaline phophatase

2 of these 4 clones were confluent in 2 plates 13 days later. IHC analysis of the 2 clones showed the expression of the pluripotency markers SSEA-1 and Oct-3/4 in both (Fig. 9). Oct-3/4 is a nuclear- and SSEA-1 a surface-marker of undifferentiated cells. In addition, AP staining was performed.

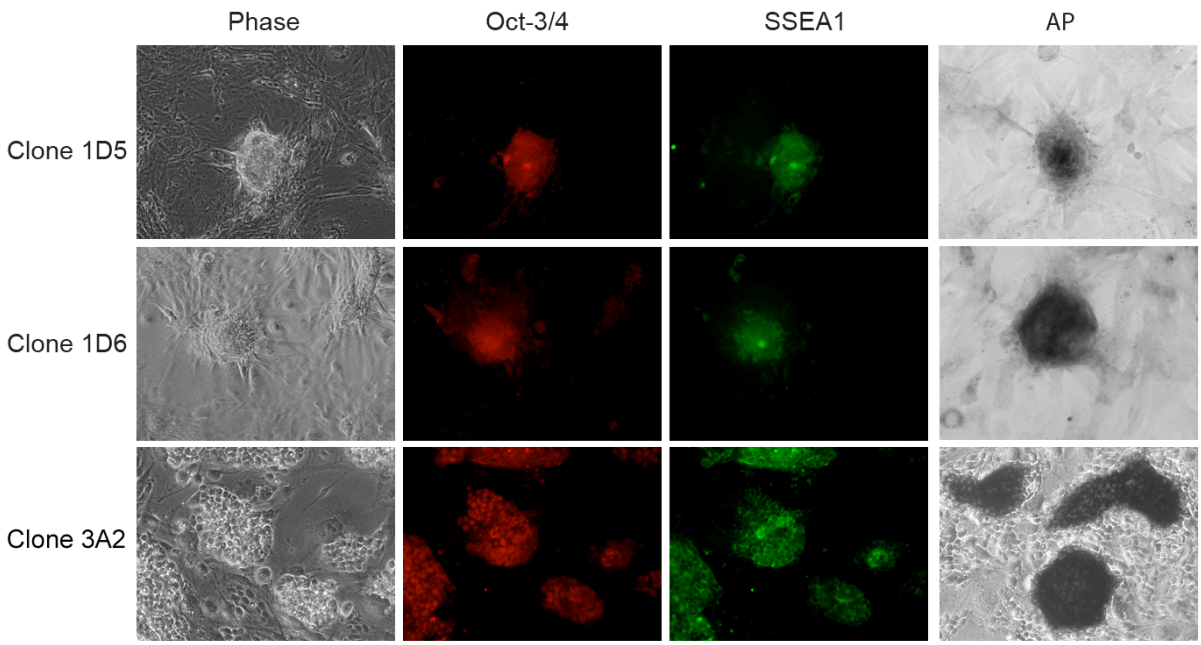


Fig. 9: IHC and AP staining from clone 1D5 and 1D6 in comparison with Clone 3A2 revealed from a earlier reprogramming of rat fibroblasts. Clones 1D5 and 1D6 exhibited expression of pluripotency markers Oct-3/4 (red fluorescence) and SSEA-1 (green fluorescence). When comparing to clone 3A2, the expression pattern of the pluripotency markers was different. Clone 3A2 exhibited a typical expression patter for Oct-3/4 and SSEA-1, while clones 1D5 and 1D6 showed a less structured and diffuse pattern. The expression for the pluripotency marker alkaline phospha-

In summary, only 4 clones out of 48 could be expanded. Compared to previously expanded iPS cell clones at the Institute, which were self-renewing within 3-5 days, the BN iPS colonies in this experiment grew slow and needed around two weeks to self renew. In addition, the staining for pluripotency revealed an unclear expression pattern, probably indicating the cells were not fully reprogrammed to a pluripotent state.

1.2.2. Expansion of KO iPS-cells

The reprogrammed KO fibroblasts showed first signs of colony formation at id 12 and were ready for being picked at id 14. 14 colonies were picked and incubated for 5 min. with trypsin and transferred into a 24 well dish. The picked clones were observed for the next days and medium was changed every second day. 10 days after picking, only for few of the 14 picked clones a colony was visible. As it was observed in the first reprogramming round, the few remained colonies showed a compact morphology and were turning darker, probably because of necrotic cells. Therefore these clones were discarded at this point.

In a second picking round, 48 clones were picked into two 24 well dishes coated with SNL feeders. Compared to the first round, this time the clones were incubated for 10 min. in trypsin, in order to dissociate single cells from the colonies, before the subsequent transfer into the 24 well culture dishes. One day after the picking, the number of colonies in each well was checked. In the most wells one colony was observed. In a few wells 2 or no colony was visible. After 6 days of observation of the 48

clones no new-formed colonies could be obtained. In the wells where no colony was present the medium was changed continuously in order to check if any new colonies will be formed in the next few days. In the wells where one or more colonies were present, the dissociation of the cells was performed by incubating the cells for 10 minutes with trypsin. To be sure not to lose some colonies during the transfer to a new 24 well culture dish, the dissociation was performed without any transfer. Interestingly, after this splitting step we could not obtain any colony in any well, indicating that the colonies were either dissociated or dead or the cells after dissociation were differentiated and not anymore distinguishable from the feeder cells.

In the following two weeks, medium was changed every three days. Then, the medium was not replaced for a period of 6 days. At this point, in 2 out of 48 wells, new colony formation could be obtained (Fig. 10).

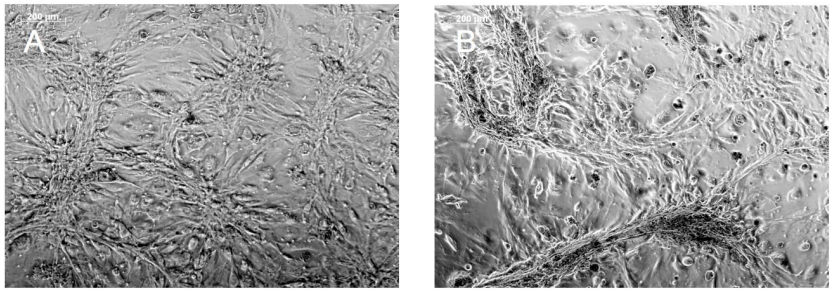


Fig. 10: New colony formation in 2 wells out of 48 wells. 25 days after picking and after 1 splitting step. **A:** Clone A5. **B:** Clone C1. Magnification: 5x

These two clones were then split at a ratio 1:1 into 3.5 cm dishes in order to expand them further. In 5 other wells colony formation was observed 6 days later and new colonies could be observed in the next days also in the other wells. Cells were then further passaged and frozen.

In summary it took only a single splitting step before the colonies seemed to dissociate, but more than 3 weeks before new colonies were formed. After this adaptation time the clones could be expanded without problems.

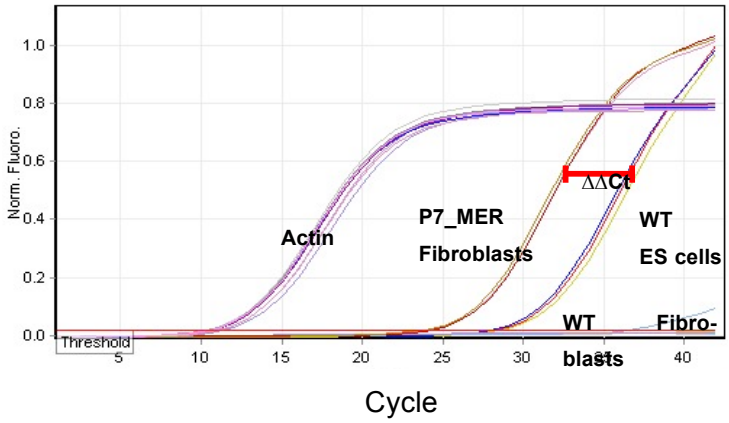
2. Analysis of Pramel 7 function in reprogramming efficiency

2.1. Quantification of genes expression levels: Real-Time PCR

For a precise quantification of transgene expression in the fibroblast cell lines overexpressing Pramel7-MER a Real-Time PCR analysis was performed. Normal fibroblasts do not express Pramel7 that is typically expressed only in the morula, in the blastocyst and in ES cells. A comparison with WT ES cells was also performed.

WT and Pramel7-MER overexpressing fibroblasts were cultivated till they were confluent, total RNA was extracted and reverse transcribed. Clone 1 was a wild type cell line, while clone 12 was a cell line carrying the Pramel7_MER construct. Both clones were derived from the same litter.

Quantitative Real-Time PCR was done in triplicates and the values were normalized to the corresponding amounts of β-actin mRNA. The fibroblasts transgenic for Pramel7_MER showed a Pramel7 overexpression that was around 16 times higher than in pluripotent wild type embryonic stem cells. As expected wt fibroblast did not show Pramel7 expression (Tab. 3).



Tab. 3: Real Time PCR graphic of WT and Pramel7_MER fibroblast compared with embryonic stem cells**.** The ΔΔCt between the transgenic fibroblasts and the ES cells means a factor 12 of Pramel7 overexpression. WT fibroblasts did not show as expected any expression of the pluripotency gene Pramel7, for this reason any signal was detected with the Real Time PCR. Actin housekeeping gene was used for the normalisation.

2.2. Reprogramming of wild type and Pramel7-MER overexpressing MEFs

To compare reprogramming efficiency of WT MEFs and transgenic mouse embryonic fibroblast (Pramel7_MER), these 2 cell types were cultivated under different culture conditions. Therefore, we reprogrammed 4x6 cm plates with WT fibroblasts and 5x6 cm plates with transgenic fibroblasts carrying the Pramel7_MER construct. For both cell types, 400 000 cells were used for the reprogramming in each plate. All cells were reprogrammed with only three factors (Oct-3/4, Klf4, Sox2). The goal of these experiments was to determine if Pramel7 overexpressing fibroblasts were able to reprogram in absence of LIF only through the overexpression of Pramel7.

The WT and Pramel7_MER cell line, derived from C57BL/6j mice, were established at the Institute of Laboratory Animal Science, University of Zürich.

The different media are either based on iPS-medium or on N2B27-medium. N2B27 is a chemically defined medium developed to support the undifferentiated growth in hES cells. The media were then supplemented with variations of LIF, OHT and 2i (Tab. 4). Reprogramming was performed on a layer of CD1-feeders, these feeders were chosen because they produce low amounts of LIF and therefore allow a better analysis of the different cultivation conditions. The classically used SNL feeders overexpress LIF and this would disturb the analysis for example of cells cultivated in absence of LIF addition to the medium.

LIF is known to prevent differentiation by activating Stat3 whereas 2i, a combination of a MEK inhibitor and a GSK3 inhibitor, is known to block differentiation. OHT is able to induce the activation of Pramel7_MER in fibroblasts carrying the Pramel7_MER construct.
The compositions of the applied media were chosen in order to determine the influence of Pramel7 overexpression in the reprogramming efficiency.
In the reprogramming procedure the cells are kept in fibroblast medium until id 3. At this point the according media was applied to each plate (Tab. 4).

Type of reprogrammed MEFs.	WT	Pramel7_MER and Stat3_MER
Culture conditions for the different reprogramming plates (1-5).	1. iPS-medium +LIF 2. N2B27 + 2i +LIF 3. N2B27 + 2i 4. iPS-medium +OHT	1. iPS-medium + LIF + OHT 2. iPS-medium + LIF 3. N2B27 + 2i + LIF + OHT 4. N2B27 + 2i + OHT 5. iPS-medium + OHT

Tab. 4: Culture conditions used for the reprogramming

For the next 12 days the different media were changed every day in all 9 plates. At id 12 first signs of new colony formation could be observed. At this point and at id 16 pictures were taken (Fig. 11-12).
At id 12 and id 16 the reprogrammed WT MEFs showed an ES cell-like morphology when cultivate under the iPS + LIF conditions (Fig. 11), whereas the cells cultivated in iPS-medium supplemented with OHT produced many non ES cell-like cells and also no colonies, as it was expected caused by the absence of LIF (Fig. 11). The WT MEFs cultivated in the plate containing N2B27-medium supplemented with 2i and LIF showed many ES cell-like colonies (Fig. 11). When the N2B27-medium was supplemented only with 2i, at id 16 only a single ES cell-like colony could be observed (Fig. 11).
The colonies obtained from the reprogrammed Pramel7_MER MEFs were difficult to categorise at this point. In the iPS + LIF conditions, as well as in the conditions when iPS-medium was supplemented with OHT, spots of small undefined colonies could be observed (Fig. 12). In the plate with the reprogrammed Pramel7_MER MEFs cultivated in iPS-medium containing LIF and OHT, areas with an accumulation of cells could be seen (Fig. 12). In both N2B27 conditions (either with 2i or with 2i+OHT) we obtained accumulation of cells, which formed fields, which were showing a morphology slightly different from the classical iPS morphology (Fig. 12), nevertheless this colonies were alkaline phosphatase positive.

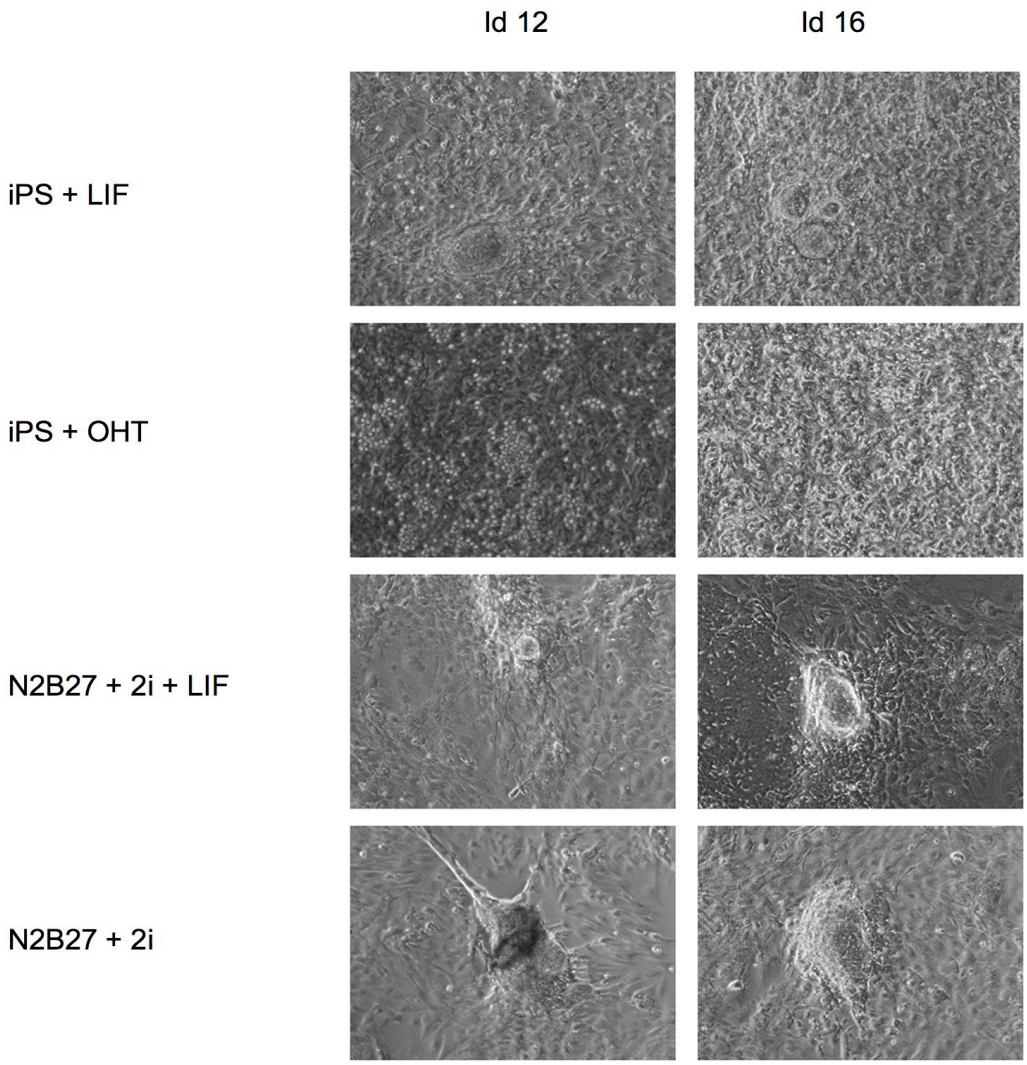


Fig. 11: WT MEFs under different culture conditions at id 12 and id 16. Magnification: 10x

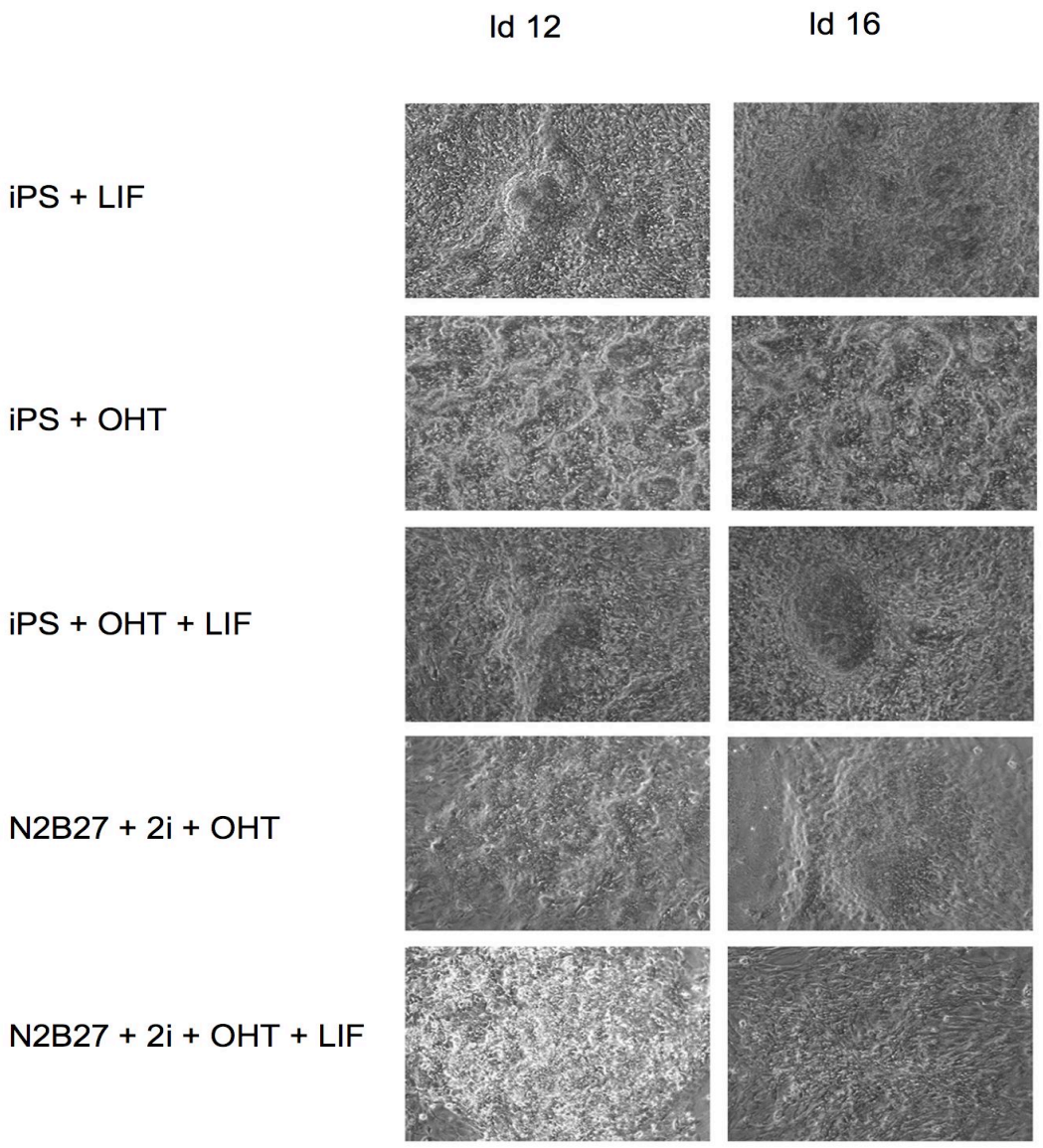
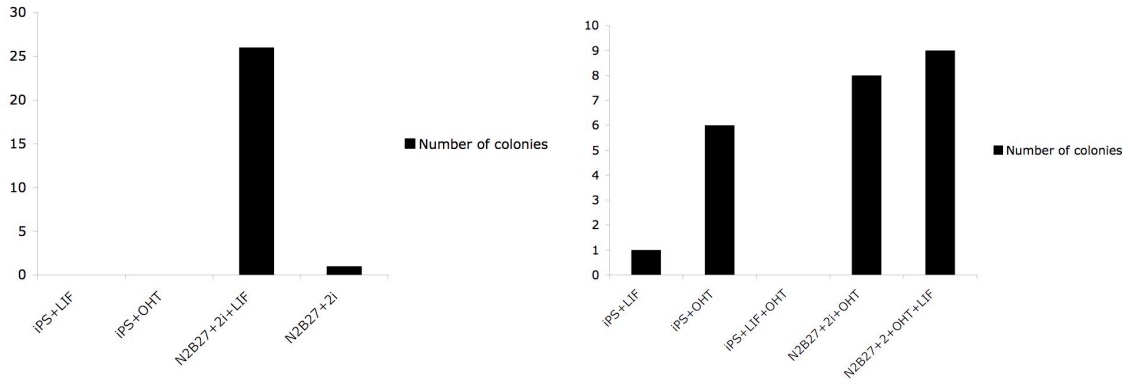


Fig: 12: Pramel7_MER MEFs under different culture conditions. Magnification: 10x

2.3. Staining for Alkaline phosphatase and counting of colonies

At id 22 the CD1-feeders started to detach and therefore the cells were fixed and stained for AP at this point. To analyse the reprogramming efficiency, the colonies of each plate, which were positive for alkaline phosphatase, were counted under the microscope and pictures were taken (Tab. 5) (Fig. 13-14).



Tab. 5: Amount of colonies obtained from the two cell types under different culture condition. **A:** Number of colonies from reprogrammed WT MEFs. **B:** Number of colonies from reprogrammed Pramel7_MER MEFs.

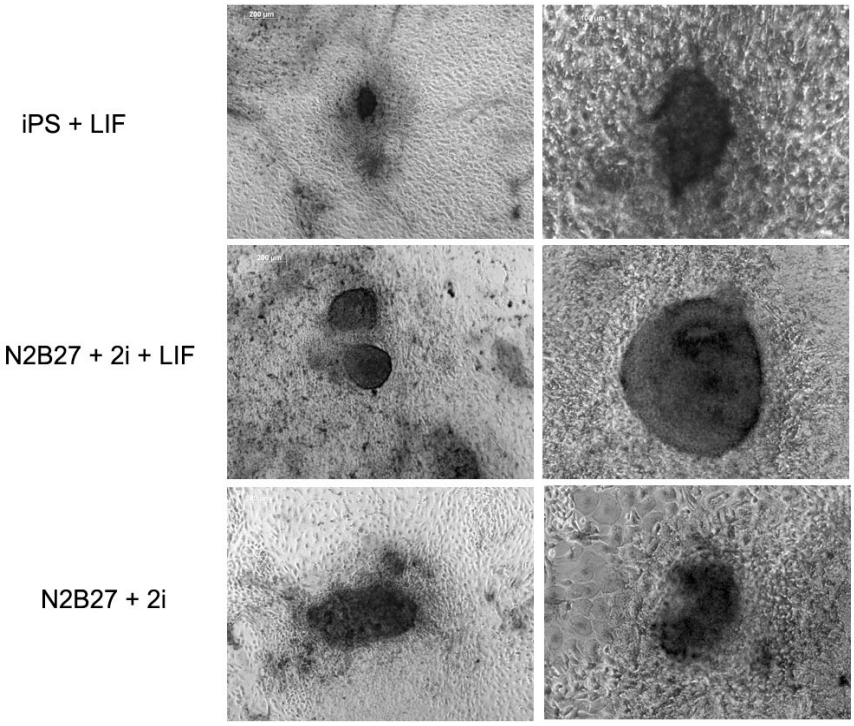


Fig. 13: AP staining for colonies obtained from reprogrammed WT MEFs cultivated under different culture conditions. Pictures are only showed for culture conditions where colonies could be seen.

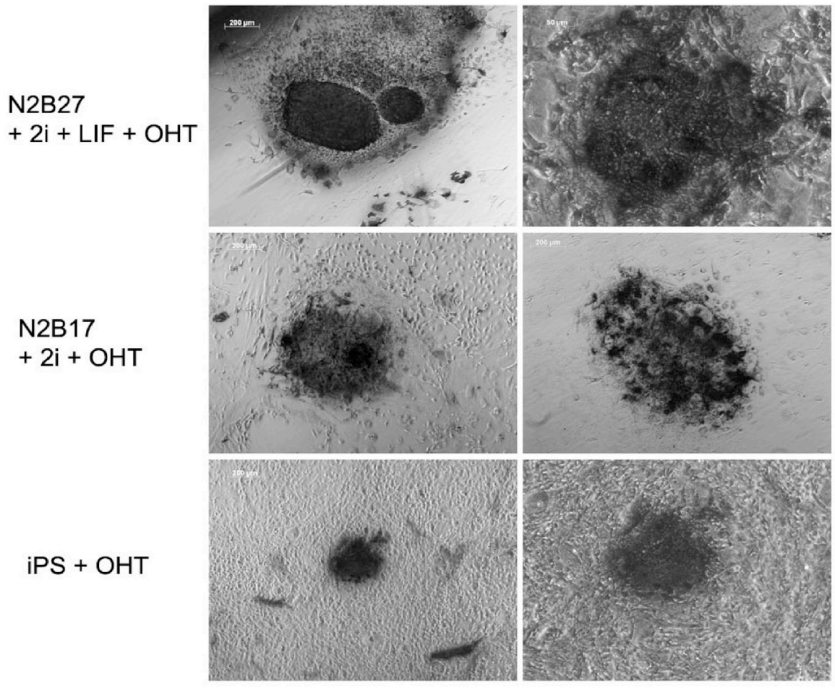


Fig. 14: AP staining for colonies obtained from reprogrammed WT MEFs cultivated under different culture conditions. Pictures are only showed for culture conditions where colonies could be seen.

E. Discussion

In 2006 Yamanaka and colleagues developed a new methodology to convert somatic cells to a pluripotent ES cell-like state. To do so, they introduced four defined transcription factors, Oct-3/4, Sox2, Klf4 and c-myc, by retroviral infection in the somatic cells. The resulting cells, termed iPS cells, have the ability to self renew indefinitely and express the classical pluripotency markers as ES cells. They further have the capacity to differentiate *in vitro* into cells from all three germ layers, to form teratomas, containing cell types from all three germ layers, and *in vivo*, to generate germline competent chimeras (Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007).

The methodology developed from the Yamanaka group opens a new field in stem cell research, not only in terms of future medical applications but also for new approaches in gene targeting in the rat.

In recent years many efforts have been done in order to gain a better understanding the molecular mechanisms of reprogramming. Even though the exact mechanisms how the 4 factors used by Yamanaka are mediating reprogramming are unknown, several genes have been described to play a pivotal role in the maintenance of pluripotency. One of these genes is the pluripotency related gene Pramel7 (Cinelli et al., 2008)

Goal of this master thesis was to clarify some of the important questions concerning the possible use of iPS cells for gene targeting in the rat and the efficiency of the generation of these cells by using different culture media. The aims of this work were:

1. Establish if differences in rat strains also influence the efficiency of reprogramming.
2. To compare the efficiency of the generation of riPS cells from wild type REFs compared with the reprogramming efficiency of the same REFs after gene targeting.
3. To examine the reprogramming efficiency between wild type mouse embryonic fibroblasts and mouse embryonic fibroblasts overexpressing an inducible form of Pramel7 (Pramel7_ MER) a protein that was recently shown to stabilize the pluripotent state of embryonic stem cells.

Reprogramming of rat fibroblast from different strains

Two reprogramming rounds were performed using fibroblast from different rat strains. In the first round, WT and KO REFs, carrying a knock out construct for neurotrypsin were used for reprogramming. Reprogramming and expansion were performed by using a SNL-feeder layer. WT and KO REFs were derived from a F1 crossing between Sprague Dawley and Brown Norway rat strains. Both cell lines were established at our Institute. In the second reprogramming round the KO REFs cell line was compared with WT REFs derived from Brown Norway rat strain. For all reprogramming procedures only 3 factors (Oct-3/4, Klf4, SOX2) were used. c-myc was omitted because of its cancerogenous properties, moreover c-myc was shown to be dispensable for reprogramming(Wernig et al., 2008).

Reprogramming and of SDxBN fibroblasts and knock out – fibroblasts

In the first reprogramming round WT and KO fibroblasts, from the same background (BNxSD), were used.

The emerging iPS colonies of WT REFs were ready for picking after the expected period of time after the infection (id 15), whereas the KO REFs were ready for picking 15-20 days later at id 34, compared to the WT REFs and to earlier experiments, which were done at Institute. That we could not observe colonies obtained from the transgenic REFs until id 34, could be caused by several reason: First, the cryotube we thawed for the reprogramming, was only containing 175 000 KO REFs. The usual amount of REFs used for reprogramming in a 10 cm dish is 800 000. This could be a reason because the reprogramming efficiency was so low. Another possibility could be that the previously performed gene targeting would have affected the capacity of these cells to be reprogrammed. This is rather unlikely because in the second reprogramming round, KO riPS colony formation was obtained at id 12. The most likely explanation is, that we missed the emerging colonies while observing the dish under the microscope. Due to fact that, the colonies were growing at the border of the 10 cm dish, they were very probably already existing before id 34. That all emerged colonies were situated at the border of the dish is very probably because the plate was shaken too much in order to distribute the REFs evenly.

Expansion of SDxBN and KO iPS- cells

Although a sufficient amount of colonies was present after the reprogramming procedure, we were not able to split and expand the riPS colonies. When the colonies were split, we hardly could observe newformed colonies indicating that the dissociation of the riPS colonies was not successful. The visible colonies after a splitting step were very dense and with the time, they were turning darker (see results, Fig. 2). Even though the riPS colonies were split several times, no increase in the number of colonies could be seen. This leads to the conclusion that the dissociation by trypsin was not sufficient to separate the cells from the very compact colonies.

The morphology of the riPS colonies obtained in the original plate during this reprogramming round was very similar to the cell morphology observed in previous experiments performed at our Institute and published by other groups (Takahashi et al., 2007). Nevertheless, soon after the first treatment with trypsin, they seemed to become necrotic and did not show self-renewal.

Even though previous works indicated that it is possible to recognize riPS colonies by their morphology, it is possible that the riPS colonies that were picked were not fully reprogrammed. Another reason for the incomplete reprogramming could be that the reprogramming of REFs on SNL-feeders results in faulty reprogrammed colonies displaying an appropriate morphology. The impaired capacity to proliferate of the picked clones could indicate that the cells were not pluripotent and therefore unable to self renew. This leads to the speculation that the dissociated cells where rather differentiating and not anymore able to form new colonies.

An explanation, that some riPS colonies were becoming dark, is that trypsinisatioin was not efficient. Therefore the colonies grew to big what possibly led to the lack of nutrients and oxygen in the centre of the colonies and subsequently to the appearance of necrotic cells.

For the KO REFs reprogramming 48 colonies were picked from the plate. In a similar way to the previously described WT iPS colonies also by the KO iPS colonies it was not possible to cultivate and expand them as single clones. As mentioned in the results section, 4 days after picking 45 out of 48 colonies, were not visible anymore. The remaining 3 colonies died within the next few days.

We assumed that the problem of expansion could be due to the fact that picking single colonies into the wells of a 24 well plate would impair the proliferation of the cells.

We therefore decided to trypsinise the whole reprogramming plate, and to observe if the colonies would show a different expansion and growth behaviour compared to the single clones.

After trypsinisation the cells were divided in 8 wells from a 24 well plate. Nevertheless, the morphology of the colonies was not changed and in none of the 8 wells signs of new colony formation could be seen. Therefore we assumed that again we were not able to dissociate the colonies to single cells. In order to optimize the dissociation efficiency we incubated the colonies for different times with either trypsin or Accutase. We decided to use Accutase, a softer dissociation reagent routinely used for ES cells, in order to be able to prolong the incubation times without killing the cells. Unfortunately incubations of 30 and 45 min. with Accutase had no effect in terms of new colony formation and we lost our cells within the next days.

As the KO REFs were reprogrammed and cultivated under the same conditions as the SDxBN REFs, the impossibility of expanding them might caused by the same reasons as mentioned for the WT REFs (incomplete reprogramming, insufficient trypsinisation and differentiation). In addition, it has to be said that KO riPS colonies were already very old when picked (id 34). If this influenced their ability to self renew cannot be ruled out. That they died because of the incubation with trypsin is possible but improbable. The duration of the trypsin incubation was only 5 min.

A further possible explanation for this inability to proliferate is that the SNL feeders do not sustain the maintenance of the riPS cells. In other earlier experiments always rat-feeders were used. Out of this we decided to use rat-feeders for a second reprogramming round.

Reprogramming of BN fibroblasts and knock out-fibroblasts

In the second reprogramming round we again reprogrammed KO and wild type REFs. While the KO cells were the same as used in the first round, the WT cells were this time derived from the Brown Norway rat strain. In order to have the KO REFs at a higher density a 6 cm dish instead of a 10 cm dish was used. The second reprogramming round was performed under the same conditions as the first one with the exception that rat feeders were used instead of SNL feeders.

Under these new conditions, we were able to observe the first signs of colony formation at id 10 for the WT fibroblasts and at id 12 for KO fibroblasts. The first colonies were picked at id 13 (WT) and id

14 (KO). Compared to the first reprogramming round, the colonies obtained from the WT REFs were picked 2 days earlier. A more clear difference was observed for the KO fibroblast. In the first reprogramming round the first colonies formation was observed at id 34. In contrast, in the second reprogramming round first growing KO colonies were obtained 22 days earlier, at id 12, and were picked at id 14. This striking different could be due either to the fact that the reprogramming was performed in a smaller cell culture dish with the same amount of REFs like in the first round of reprogramming or, what is more probable, because a rat-feeder layer was used.

The morphology of the resulting riPS was similar to the one from the first reprogramming round. The riPS colonies generated by using rat-feeders for reprogramming showed better properties in terms of the expansion ability. This will be discussed in the next paragraph.

Expansion of WT BN and KO iPS cells

For the WT REFs out of total 48 picked colonies we were able to bring only 2 clones to confluence in 3.5 cm dishes. These 2 clones were then split 1:2 in 2 dishes of the same size, and although the expanding efficiency was very low, we finally could expand these clones. For the trypsinsation procedures we used an incubation time of 10 min. because it turned out to be more successful for dissociating the colonies. The clones were then stained for the pluripotency markers Oct-3/4, SSEA1 and alkaline phosphatase. Oct-3/4 is present in the nuclei of the cells whereas SSEA1 is a cell surface protein.

Unfortunately, our staining did not show the expected pattern even though the colonies showed riPS-like morphology. The staining for Oct-3/4 showed that these cells had a weak expression of this pluripotency marker. The pattern observed in the SSEA1 staining did not show the typical expression pattern and it was difficult to say if these cells were positive for this pluripotency marker (see results, Fig. 8). A reason because the staining did not give clear results could be due to the morphology of the colonies. As mentioned before the colonies were difficult to trypsinise because they were too dense and therefore also the antibodies could not bind properly.

For the AP staining the cells showed only after a long incubation time (5h), a staining for this pluripotency marker (see results, Fig. 8).

In contrast to the first reprogramming round, in this second round the WT riPS colonies did not become necrotic after being split and could be expanded.

However these cells did express pluripotency markers, it has to be clarified if they were fully reprogrammed to an undifferentiated and pluripotent state or not. More experiments have to be done in order to check if these cells express other pluripotency markers like Nanog or Stat3.

As we assumed for the reprogramming efficiency, we also think that the rat-feeder layer had an influence on morphology and expanding behaviours of the WT iPS cells. Probably rat-feeders sustain better the reprogramming of riPS cells instead of SNL-feeders because we were able to expand riPS better when they were reprogrammed on rat-feeders instead of SNL-feeders.

For the expansion of KO iPS-cells, we performed 2 picking rounds. In the first picking round, we incubated the colonies for 5 min. in trypsin before plating them into a 24 well dish. But we observed either no colonies or the present colonies never became bigger. We could not dissociate and expand further these clones. Therefore we decided to pick new colonies from the original reprogramming plate. This time we incubate the colonies for 10 min. with trypsin before transferring them into the 24 well culture dish. Nevertheless also after several times of trypsinisations most of the colonies were still intact. After last splitting procedure it took 19 days before some new-formed colonies were observed.
Nevertheless, it was possible to expand these KO iPS cells a little bit further compared to the first reprogramming round.
The 48 KO riPS colonies obtained from a second picking round were cultivated on SNL-feeders from passage 0 on. When compared the expansion efficiency between the BN-iPS cells, which were on rat-feeders until passage 1, and the KO-iPS cells, which were reprogrammed on rat feeders only, we saw that in both cases the expansion efficiency was similar. Out of this it can be assumed that the rat-feeders contribute in the reprogramming of REFs and that they are dispensable for their expansion.
However, neither the WT nor the KO riPS cells showed normal iPS-like growth behaviour. It is known that undifferentiated ES cells have a very short cell cycle and therefore they usually need to be passaged at least every second day. Fully reprogrammed iPS cells show a similar behaviour what could never be observed with our riPS cells.

Influence of the strain background in reprogramming of REFs

Murine ES cells of proven ability to colonise the germ line have been obtained at very low frequency in only a few mouse strains other than 129 substrains (Simpson et al., 1997). Only after adjusting culture conditions, germ line competent inbred ES cells could be established, e.g. from C57BL/6(Ledermann and Burki, 1991), DBA/1lacj(Roach et al., 1995), and BALB/c mice (Kawase et al., 1994; Noben-Trauth et al., 1996).
These strain limitations observed by the mouse ES cells could also be present in rats' iPS.
In this work we reprogrammed WT inbred BN and WT hybrid SDxBN REFs. We could not see any difference in the reprogramming efficiency regarding the genetic background. Both REFs types generated colonies after the infection with the 3 factors. The further expansion of both WT inbred BN and WT hybrid SDxBN REFs was anyway not possible. In a previous work carried out in our Lab, (Fabienne Weber, 2008) the generation and expansion of riPS cells derived from Wistar outbred rat strain was successful. These outbred riPS cells showed an ES cell-like self-renewal and expressed the typical pluripotency markers.
A reason because we were not able to generate proper riPS out of BN inbred embryonic fibroblasts could of course be due to the genetic background of REFs. Interestingly we got similar results also with the hybrid SDxBN REFs. It has been showed that a hybrid genetic background stabilise the cells making them a good tool for gene targeting. Because of time restriction we were not able to repeat the

experiment, but if also future experiments will give the same results, one has to consider that maybe this background is a problem. The molecular mechanisms linked to the generation and stabilisation of riPS cells are little known to date. Therefore it is important to investigate on the molecular basis of pluripotency in rat embryonic stem cells in order to improve the generation of riPS from different strains.

Analysis of Pramel7 function in reprogramming efficiency of mouse embryonic fibroblasts

In previous studies carried out at our Institute it was possible to generate germ line competent ES cell from the non-permissive FVB/N mouse strain. In these cells, which overexpressed a mutated Stat3 fusion protein, Pramel7 was upregulated (Cinelli et al., 2008). In the same study it was shown that an overexpression of Pramel7 was sufficient to maintain ES cell undifferentiated in a LIF independent manner. These results suggest a role of this gene in maintaining pluripotency in embryonic stem cells.
Therefore, it was interesting to see if Pramel7 plays a role in the reprogramming of fully differentiated cells. For this reason, we planned to reprogram MEFs carrying the same construct used in the Cinelli et al. (2008) work but the Stat3 gene was replaced by Pramel7.
We then reprogrammed WT and Pramel7_MER MEFs under different culture conditions (Tab. 1).

Type of reprogrammed MEFs.	WT	Pramel7_MER and Stat3_MER
Culture conditions for the different reprogramming plates (1-5).	5. iPS-medium +LIF 6. N2B27 + 2i +LIF 7. N2B27 + 2i 8. iPS-medium +OHT	6. iPS-medium + LIF + OHT 7. iPS-medium + LIF 8. N2B27 + 2i + LIF + OHT 9. N2B27 + 2i + OHT 10. iPS-medium + OHT

Tab.1: Culture conditions for the different reprogramming plates.

By cultivating WT and transgenic MEFs in iPS-medium with LIF, the reprogramming efficiency should be the same whereas by adding OHT to the iPS-medium, the transgenic Pramel7 is activated and can therefore cause a difference in the reprogramming efficiency between WT and transgenic MEFs. Unexpectedly we observed iPS colonies neither from the WT nor the transgenic MEFs, under iPS + LIF conditions. This could be due to the fact that the CD1-feeders secrete LIF at a very low level compared to SNL-feeders, which overexpress LIF and are usually used for reprogramming. Under the iPS + OHT conditions, no colony was observed for the WT MEFs whereas 6 colonies emerged under the same conditions for the transgenic MEFs, which indicates that Pramel7 can compensate for LIF. As expected, WT MEFs only formed colonies if LIF was added to the N2B27 + 2i conditions. In contrast, the transgenic MEFs reprogrammed under the same conditions plus OHT, showed no difference in the number of colonies seen, irrespective of presence of LIF. This supports the assumption that Pramel7 is able to counterbalance for LIF.

Moreover we observed a higher reprogramming efficiency for both WT and transgenic MEFs, when cultivated in the N2B27 conditions compared to the iPS conditions. This could be again due to low LIF-expression of the CD1-feeders.

In summary, the experiment showed interesting results. It could be demonstrated that Pramel7 is able to counterbalance for LIF.

Due to the fact that colonies were formed neither from the WT nor the transgenic MEFs under the iPS + LIF conditions, the experiment should be repeated by using SNL-feeders in order to see if this is caused by the CD1-feeders. This could indicate that the addition of LIF to the medium is not sufficient to reprogram the cells and that the concentration should be increased in absence of the SNL feeders.

Outlook

Generation of iPS cells out of different rat strains

Due to ethical issues concerning the use of human embryos for derivation of ES cells, the development of iPS cells from somatic cells in 2006 by the Yamanaka group, was an important step in terms of clinical applications. Moreover this technique also offers new possibilities to establish gene targeting in the rat.

The experiments performed in the frame of this work indicate that the efficiency in generating and maintaining iPS from rat embryonic fibroblast is not only depending from the culture conditions but also from the genetic background of the used embryonic fibroblasts. Due to the short time of the thesis it was only possible to perform preliminary experiments and therefore many questions are still open. The following experiments should be performed in order to better clarify the effect of the cultivation conditions and the genetic background on the efficiency of iPS cell establishment:

- Repeating the reprogramming of inbred BN fibroblasts but at the same time reprogram also the outbred Wistar fibroblasts (which could be successfully reprogrammed and expanded at our Institute). It would be interesting to see if by using the same culture conditions, there are any differences in the generation and maintenance of these two different cell types.

- The generation of genetically modified rat iPS also did not completely succeed. Regarding the importance of the rat as models for human diseases, more experiments have to be done in order to establish KO iPS cells. Especially interesting would be the comparison between the generating of knock out fibroblasts by gene targeting followed by the reprogramming iPS cells with gene targeting on already reprogrammed iPS cells.

- The hybrid BNxSD fibroblasts could not be reprogrammed. It could be a problem of the background. Repeating the experiments would help to better understand why we were not able to generate iPS out of these cells. Maybe other hybrid cells could be better reprogrammed than BNxSD and generate more stable cells.

- The striking difference between cultivation with SNL feeders and rat fibroblasts should also be further analyzed. Interestingly, mouse fibroblasts can only be reprogrammed in presence of an overexpression of LIF through the feeders. Nevertheless, reprogramming of rat fibroblasts on this type of feeders cells, does not work efficiently. It is therefore to assume that rat fibroblasts secrete, or express on their surface, factors that sustain the reprogramming process.

Analysis of Pramel7 function in reprogramming efficiency of mouse embryonic fibroblasts

The newly discovered pluripotency gene Pramel7 was shown to play an import role in the maintenance of pluripotency in embryonic stem cells. For this reason one of the goals of the following work was better characterize its function during reprogramming. This properties were analyzed by cultivating mouse embryonic fibroblasts carrying a Pramel7_MER fusion protein under different culture conditions. Our results are quite interesting but also in this case do to the short time available it will be necessary to further examine the role of this gene in reprogramming by performing more experiments:

- Due to the fact that in our work we were not able to generate WT iPS under the common condition (iPS medium supplemented with LIF) it would be important to clarify if it was a problem of the feeders we used for reprogramming and cultivating these cells. It would be of advantages to repeat the experiment by using SNL feeders instead of the CD-1 we used in this work. This would further stress the importance of LIF during reprogramming, and define LIF as a "reprogramming factor" at the same strength like the Yamanaka factors.

- The data obtained in this work indicate that the induction of Pramel7 by OHT is sufficient to induce the generation of iPS colonies, even in absence of LIF. The state of the obtained iPS cells has to be better characterized, especially their pluripotency has to be further analyzed by testing the ability of theses cells to generate teratoma containing cells from all three germ layers and finally their capacity to contribute in the generation of germline competent chimeras. This would give a clear sign of the importance of Pramel7 not only in maintaining pluripotency but also in the generation and stabilization of iPS cells.

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Lebenslauf

Name	Jon Paulin Zumthor
Geburtsdatum	12.Januar 1982
Geburtsort	Chur
Nationalität	CH
Heimatort	Haldenstein, GR

1989 – 1995	Primarschule, Haldenstein
1995 – 1998	Sekundarschule, Chur
1999 – 2003	Bündner Kantonsschule, Chur
2003	Matura an der Bündner Kantonsschule, Schwerpunktfach Wirtschaft, Chur
2004 – 2010	Studium der Veterinärmedizin an der Vetsuisse Fakultät Universität Zürich
2010	Staatsexamen der Vetsuisse Fakultät Universität Zürich

26. Oktober 2010

Jon Paulin Zumthor